Conventional and Novel Treatments for Control of Clubroot Disease of Brassicas

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Abstract

The aim of this project was to develop treatments that would be able to reduce the survival of clubroot spores in field soil and protect the roots of young transplants against infection. The project focused on using combinations of treatments which integrate novel and existing controls with emphasis placed on the sustainable use of waste materials, plant materials and bioactives. A wide range of treatments were screened individually and in combination under glasshouse and field conditions, e.g. fungicides, nutritional amendments, companion planting, plant saponins and biocontrol agents.

Many of these treatments were able to reduce clubroot severity to varying degrees. Glasshouse treatments were more successful at controlling disease than those applied in the field. The most effective treatments - when applied correctly - contained calcium, e.g. lime as calcium oxide or LimeX (a by-product of the sugarbeet processing industry), and crushed scallop and whelk shells (a by-product of the fishing industry). Whilst the effects of calcium and pH on clubroot are not new, growers need to think more in terms of dose of calcium applied in the field rather than just pH, and also, the time of addition of lime to soil before planting needs serious consideration as it may be optimal to apply lime less than two weeks before transplanting.

Experimental results have shown that soil microflora plays a major role in the development of clubroot disease and that the membrane potential of growing roots may be one of the most important factors in preventing *P. brassicae* from entering plant roots and causing disease due to the effect that calcium and pH have on clubroot control. The experiments have also shown that there are interactions between treatments such as fungicides, limes, soil nutritional level and soil microflora at controlling disease and some treatments may reduce the effectiveness of other treatments at controlling disease.

Another factor that is known to affect the effectiveness of treatments at controlling clubroot is the initial spore load in the soil. Therefore, an additional aim of the project was to develop a rapid, quantitative PCR based diagnostic test that could measure the

level of clubroot spores directly from soil. *Plasmodiophora brassicae* DNA was successfully extracted and amplified from artificially inoculated soils and from naturally infested field soils using real-time PCR with selected sets of primers and probes. Many different types of soil DNA extraction methods were tested and standard curves relating to different levels of spore inoculum were created.

This project has generated useful information as to why there are contradictory results in clubroot research about the effect of various treatments at controlling clubroot. This information may also be the basis of practical advice to brassica growers on best practices to use to achieve optimal clubroot control in the field. Options in relation to new sustainable control treatments are discussed in the light of the results from both glasshouse and field experiments. These involve planting brassicas on raised beds and applying treatments strategically around the root zone. LimeX 70 or powdered calcium oxide were demonstrated to be the most optimal lime treatments for control, and a split application of Perlka (granular calcium cyanamide) may prove to be a consistently effective method for controlling clubroot.

Declaration

I hereby declare that this thesis is my own composition, that the work described is my own except where assistance is explicitly acknowledged, and that it has not been submitted for any other degree or professional qualification.

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Table of Contents

List of Figures

List of Tables

CHAPTER 1 – BIOLOGY AND CONTROL OF PLASMODIOPHORA BRASSICAE

Introduction to *P. brassicae* and clubroot

Clubroot is caused by the soil-borne Protist *Plasmodiophora brassicae*, an obligate parasite that causes one of the most damaging diseases of vegetable brassicas in the world. Historical reports of clubroot date back to the thirteenth century in Europe where it was called 'finger and toe' disease because of the shape of infected roots (Miller *et al*., 2004). *Plasmodiophora brassicae* can be found worldwide in all temperate zones and infects over 300 species in 64 genera of both cultivated and wild crucifers (Grabowski, 2004). Economically important hosts of *P. brassicae* include turnip, cabbage, cauliflower, broccoli, Brussels sprouts, Chinese cabbage and radish. The primary phases of colonization have also been recorded on some non-cruciferous hosts including members of the Poaceae, Rosaceae and Papaveraceae (Donald *et al.,* 2006). It is estimated that clubroot causes £30 million in crop losses in the UK each year and causes between 10 to 15 percent of crop losses worldwide (Donald *et al.,* 2006). Despite the fact that this organism causes such large economic losses and has been researched for many years, there are still significant gaps in the knowledge of the biology and therefore the control of clubroot disease.

Aims of the project

The major aim of the project was to evaluate the usefulness of novel treatments at controlling clubroot either as sole amendments, in combination, or in conjunction with appropriate existing control measures. These novel treatments were developed for field use as a more adequate and sustainable way to control clubroot than the current methods. An additional aim of this project was to develop a quantitative PCR diagnostic test to quantify the levels of *P. brassicae* present in soils. This would help brassica growers to select cost-effective treatments for controlling the specific level of disease in their fields. It would also provide a research tool for examining the effectiveness of treatments at reducing spore inoculum in the soil.

Clubroot symptoms

The first observable above ground symptom of clubroot is often daytime wilting. Otherwise healthy looking plants wilt on hot dry days, recovering once the sun sets or temperatures cool. As the disease progresses, leaves yellow and die. Diseased plants are stunted compared to uninfected plants. The most characteristic symptom of *P. brassicae* infection is the abnormal enlargement (clubbing) of host roots - hence the name of the disease. Fine roots, secondary roots, the taproot or even the underground stem of certain brassica species can be abnormally enlarged. Clubs on the roots may be isolated and only cover part of some roots, or they may coalesce and cover the entire root system of the plant. Yield and quality of the infected crops can be severely affected by clubroot, as the deformed roots are unable to absorb water and nutrients effectively. Clubbed roots can develop secondary rots that can cause the breakdown of the whole root system.

Taxonomy of *P. brassicae*

For decades, *P. brassicae* was classed as a plasmodiophorid. More recently, Braselton (1995) has suggested that *P. brassicae* should be placed in the protozoan grouping, describing them as a monophyletic group of uncertain systematic affinities. Unusual characteristic features of *P. brassicae* include closed mitosis known as cruciform nuclear division, the presence of a Rohr and Stachel – a cellular protrusion used by the zoospores in the infection of host cells, obligate intracellular parasitism, the formation of multinucleate plasmodia inside their hosts, biflagellated, heterocont zoospores and environmentally resistant resting spores. Recently, molecular evidence based on the sequence of actin genes has suggested that *P. brassicae* is related to Cercozoa and Foraminifera (Archibald and Keeling, 2004).

Life-cycle of *P. brassicae*

The life cycle of *P. brassicae* is not fully understood despite many years of research. This may, in part, be because the organism is an obligate parasite which needs a host plant to complete its life cycle and there is no known way of culturing the organism

artificially (Niwa *et al.,* 2007). It is generally agreed that the life cycle has two stages: the root hair phase (primary stage) following root hair infection; and the cortex/stele phase (secondary stage) following cortical infection (Ingram and Tommerup, 1972). It is essential to improve the understanding of the life cycle of *P. brassicae* as this knowledge could provide insights into ways that the disease may be controlled.

Resting spores

P. brassicae resting spores are spherical, have a diameter of approximately 3–5 µm and possess a spiny wall that has a granular matrix spread over it (MacFarlane, 1970). Resting spores are haploid with a single nucleus of 1.5 µm diameter in the centre of the spore. The resting spores of *P. brassicae* are extremely robust. Field studies carried out by Wallenhammar (1996) suggest that resting spores have a half life of 3.6 years and that it requires at least 18 years in the absence of a suitable host for populations to be reduced to levels low enough not to cause disease. A concentration of 1000 spores/g soil has been shown to be needed for symptom development to occur (Wallenhammar, 1996). The resting spores of *P. brassicae* can be spread from field to field by infested soil, contaminated water supplies, infected transplants, infested soil on farm machinery, and by animals.

The robustness of these resting spores is attributed to the cell wall characteristics (Moxham, 1983). The cell wall is the interface of the spore with the environment and is the barrier that potentially harmful biological or chemical agents must penetrate in order to have an effect. Therefore information on the chemical and morphological make-up of this wall could be valuable in the development of control measures. The resting spore cell wall is composed of 25% chitin, 2.5% other carbohydrates, 34% proteins and 18 % lipids (Moxham, 1983). Protein is therefore the main component of the cell wall and the majority of this protein is alkali soluble. The protein and lipid content is very high compared with spores of other microorganisms. These unique wall attributes may explain why the spores survive so long in the soil and are apparently resistant to biocidal agents. The spore wall is spiny but has an exit pore from which the zoospore emerges

(Tanaka *et al.,* 2001). There is a fibrous layer on the spines which is made up of the non-granular constituents of the interspore matrix. This may explain why spores clump together in solution.

The germination of resting spores requires moist soil (Monteith, 1924) and can occur over a wide temperature range of 12°C - 27°C (Chupp, 1917). Although clubroot has been found in soils exhibiting a wide pH range from 4.5-8.1, the disease is primarily associated with acid soils which may promote germination (Colhoun, 1953). Many other factors have been shown to promote resting spore germination (Kowalski and Bochow 1996) and will be discussed later. When soil conditions dictate, the resting spores of the pathogen germinate, a process which takes around 18 h (Wellman, 1930). A single naked zoospore protoplast emerges from a circular pore (1.4 µm) in the wall of each germinating spore. Two unequal anterior flagella appear almost immediately after the cytoplasm emerges from the pore (Ellison, 1945). Once liberated, the zoospore protoplasts are slightly ovate and between 3-5 µm in diameter. These primary zoospores "swim" by means of their flagella to infect susceptible plant root hairs. The number of germinating spores increase over a number of days indicating that spores do not all germinate en masse but germinate at different times (Ellison, 1945).

Primary zoospore penetration of root hairs

This account of primary zoospore penetration of root hairs is based on work carried out by Williams *et al.,* (1971). Zoospores collide with root hairs several times before they become attached to one. The point of attachment is on the side of the zoospore opposite the point of flagellar insertion. After a primary zoospore attaches to a cabbage root hair wall, the flagella coil around the zoospore body, which becomes slightly flattened against the host wall. The flagella may continue to beat for a while then become quiescent. During the next 2 hours a bullet-like stachel is formed within a tubular cavity in the cyst, the Rohr. Possibly because of pressure on the expanding cyst vacuole, the Rohr is quickly evaginated to form a bulbous adhesorium. The stachel punctures the host wall, and then in about one second, the parasite is injected into the root hair. The

time required for adhesorium formation and host penetration is about one minute. After penetration, a callose-rich papilla is soon formed between the host wall and plasma membrane at the penetration site. Young amoebae then develop in the root hairs. They have no lipid bodies and are surrounded by 7-layered envelopes.

Development of primary zoospores into secondary zoospores in the root hair

Root hairs become infected by primary zoospores within one day of primary zoospore emergence and the uninucleate, membrane bounded, primary plasmodia undergoes a number of synchronous nuclear divisions to become multinucleate soon after entering the host cytoplasm (Ingram and Tommerup, 1972). Root hairs can contain more than one plasmodia but no fusion has been observed between them (Ingram and Tommerup, 1972). The plasmodia enlarge and the number of nuclei increase until at six days after infection, many root hairs become completely filled with plasmodia, some containing 50-100 nuclei (Ingram and Tommerup, 1972). The initial stages of zoosporangium formation inside primary plasmodia is observed after the third day (Ingram and Tommerup, 1972). At 8 days, most plasmodia have undergone sporogenesis. It is thought that secondary zoospores released from these sporangia either re-infect root hairs or fuse in pairs and infect cortical cells; the latter leads to the formation of clubbed roots (Ingram and Tommerup, 1972). Active secondary zoospores are not observed until the third day, and within three hours of their release, cortical infection occurs, hence the view that secondary zoospores are necessary for cortical infection (Ingram and Tommerup, 1972). A number of clubroot researchers have claimed that secondary zoospore fusion is required for cortical infection however, no one has properly observed the fusion process taking place or clerly identified the cause of cortical infection (Voorrips, 1996).

Infection of the cortical cells by secondary zoospores

Research has shown that no cortical infection by secondary zoospores has been observed at less than -150 mbars in silt and sandy loam soils (50-70% water-holding capacity), but it has been found at -200 mbars in a high organic matter soil (Thuma *et al.,* 1983). A

matric potential of -150 mbars corresponds to soil pores up to about 20 µm in diameter being filled with water (Thuma *et al.,* 1983). The diameter of clubroot zoospores is about 3 µm (Thuma *et al.,* 1983). This suggests that soil moisture content could control the development of disease depending on soil type.

Development of the organism inside the cortex

Plasmodiophora brassicae development inside the cortex can be separated biochemically into two stages; active metabolite accumulation and metabolite degradation which leads to gall formation – the characteristic symptom of clubroot disease (Williams *et al.,* 1974). Initial cortical infection is accompanied by rapid vegetative growth of *P. brassicae* with increasing DNA, RNA, protein, lipid, amino acids, starch and sugar levels (Williams *et al.,* 1974). This results in the enrichment of host cytoplasm in ribisomes, mitochondria, dictyosomes and starch grains. Gall formation in many plant-microbe interactions can be triggered by alterations in either auxin/cytokinin metabolism (Devos *et al.,* 2005). Although the importance of these hormones in clubroot infection has long been recognised, the literature contains contradictory data on the plant hormone content of the root during clubroot infection. During clubroot formation a higher level of indole-3-acetic acid (IAA/auxin) can be detected in infected roots compared with uninfected roots. The timing of when this increase occurs is contradictory throughout the literature with increases in auxin content in roots recorded at 17, 20, 25, 30, 36, 40 and 60 days after infection (DAI) (Devos *et al.,* 2005), but Ludwig-Muller *et al.,* (1996) suggested that the substantive increase in auxin in plant tissues occurs at 10 and 12 DAI. This concurs with when the organism is developing inside the cortex and indicates that there may be a role for auxin in the development during the secondary life cycle stage of *P. brassicae*. Data published on the cytokinin content of roots are more consistent with far more cytokinin activity (because of the increased cytokinin content) in infected roots than in healthy roots (Devos *et al.,* 2005). Infected plants are cytokinin-independent for their growth in tissue culture and isolated secondary plasmodia of *P. brassicae* can take up ^{14}C-adenine *in vitro* and incorporate it into trans-zeatin, a pre-cursor of cytokinin (Devos *et al.,* 2005).

Therefore it may be inferred that the plasmodia can at least partly synthesise cytokinins and this may play a role in the development of clubroot gall symptoms. Apart from the increase in auxin and cytokinins during the synthetic phase of club formation, the activities of starch synthetases also increase (Williams *et al.,* 1974). Fatty acid synthetase activity is also greatest during the synthetic phase of the *P. brassicae* life cycle (Williams *et al.,* 1974). This would this suggest that lipids are important in the development of *P. brassicae*.

Just prior to the degradative phase of the life cycle, an increase in the activities of α and β amylases and phosphorylase occur along with increased lipase activity (Williams *et al.,* 1974). During the degradative phase, sugars, RNA and starch levels decrease but protein, amino acids, DNA and lipids remain high (Williams *et al.,* 1974). The galls forming from the uncontrollably dividing host tissues become major sinks for carbohydrates (Evans and Scholes, 1995) and this contributes to the yield loss in the infected plants. The plasmodia also cause the vascular tubes to distort, causing water and nutrient transport to be affected, also leading to the yield loss. This stage of the life cycle is therefore very important in the control of clubroot because it is this stage of the life cycle that causes the root galling and adverse effects on plant growth and nutrition.

Sporogenesis

An account of sporogenesis is summarised by (Williams and McNabola, 1967). They saw that the vegetative plasmodium of *P. brassicae* was contained within host cell cytoplasm. The absence of any necrotic response by the host cytoplasm is a reflection of the high degree of compatibility between the host and parasite. The multinucleate plasmodium contains numerous large lipid droplets and is full of mitochondria, dictyosomes, endoplasmic reticulum (ER) and ribosomes (Williams and McNabola, 1967). The first indication of the transition from vegetative growth to sporogenesis is the reduction in the thickness of the plasmodial envelope. The outer membrane of the plasmodial envelope separates from the inner membrane leaving each plasmodium bounded by a single membrane – the plasmodial membrane. Nucleoli disappear and

numerous vacuoles (formed by the invagination of the plasmodial membrane) and vesicles (many containing a granular matrix) appear in the cytoplasm of the plasmodium. These vacuoles become aligned in planes of cleavage around each nucleus forming the boundaries of each future sporangium. As these vacuoles coalesce, the nucleus of each young resting sporangium becomes surrounded with cytoplasm and is enveloped by the vacuolar membranes. Spines form on the sporangial membranes as aggregates of residual vacuolar material. The sporangial wall is then deposited between the sporangial membrane and the spines. Intact host nuclei, mitochondria and plastids can be found dispersed among the mature resting sporangia within vacuoles. Host sterols are also thought to be used by *P. brassicae* as a storage energy source (Knights, 1970). Ito *et al.,* (1999) have also suggested that host DNA is present with the *P. brassicae* genome. Eventually, due to microbial degradation of the roots, the resting spores that have been formed are released into the soil as a new source of inoculum for infecting new hosts. One plant can produce 10^{11} new resting spores (Narisawa and Hashiba, 1998). If a way could be found of preventing the formation of new resting spores, this would reduce the number of resting spores released into the soil.

Summary of the effects on host plants of *P. brassicae* infection

Infected plants show a transient growth promotion early in infection from 3 DAI to 13 DAI (Devos *et al.,* 2005). There is an increased total auxin pool and also a xyloglucan endotransglucosylase/hydrolase increase in the roots. The increase in these two substances results in wall loosening and therefore cell expansion. When the first secondary plasmodia are formed, increases in host synthetic activity suggest an enhanced host metabolism (Williams and McNabola, 1967). Gall formation occurs after 14 days in the upper root system (Devos *et al.,* 2005) and maintenance of the meristematic condition appears to be the chief response of the host cell to the parasite (Williams and McNabola, 1967). Active cytokinins are higher in infected plants compared to non-infected plants (Devos *et al.,* 2005). Auxin conjugates are also much greater in infected plants than in control plants early on in infection but after 21 days there is a lower level of auxin in infected plants compared with control plants reflecting

an auxin sink (Devos *et al.,* 2005). The involvement of auxin and cytokinin is therefore thought to play a role in club development by causing uncontrolled cell division and expansion. Massive changes to host metabolism accompany gall formation, with increases in protein, DNA, RNA, amino acids, starch and lipids in infected roots. These increases are reflected by the enrichment of host cytoplasm in ribosomes, mitochondria, dictyosomes and starch grains (Williams and McNabola, 1967). The galls forming from host tissues become major sinks for carbohydrates and so plant growth slows. As sporogenesis occurs, there is a rapid loss of host starch from the amyloplasts (Williams and McNabola, 1967). The degradation of starch could reflect the inability of the parasitized cell to provide adequate energy precursors and carbon for continued vegetative parasite growth, thus inducing sporogenesis (Williams and McNabola, 1967). As sporogenesis occurs, starch and sugar contents decrease threefold within the host; whereas DNA, RNA and lipid remain constant (Williams, *et al*., 1974). The host ribosome then breaks down. The degeneration of the outer membrane of the envelope at the same time as host ribosome breakdown suggests that the integrity of this outer membrane may be under host control (Williams and McNabola, 1967).

Differences between resistant and susceptible plants

If the host plant is resistant to *P. brassicae* (i.e. cultivars with genetic resistance) then there are differences in the response of the host plant. In plants termed resistant, only the primary stage of the life-cycle takes place indicating that it is likely that it is the secondary stage that is required for the clubbing symptoms of clubroot (Ludwig-Muller *et al.,* 1997). Takahashi *et al.,* (2006) observed that resistant roots cause an alkalisation of the root zone whereas susceptible roots do not. They also discovered that there is a hypersensitive response (HR) in resistant plants only. The programmed cell death caused by the HR may restrict the organism from moving into the cortex and this may be why the clubbing symptoms do not occur in resistant plants.

Several metabolic differences between resistant and susceptible plants have been identified by Graveland *et al.,* (1992). There are ten times the level of starch produced

in susceptible plants compared to resistant plants. The quantity and type of starch build up are related to the vigour of the host plant. Non parasitised cells of actively growing resistant plants build up crystalline amylose, whereas infected cells may also synthesise large quantities of amorphous amylopectin. In aging plants, infected cells produce mainly amylose. The appearance of new forms of parasite-induced host substrate points to at least a partial control of host metabolism by the parasite. There is also up to a ten-fold increase in glucose ^{14}C incorporation that occurs in parasitised cells over that which occurs in non-infected susceptible cells. This indicates that an altered host metabolism may have an effect on clubroot development.

During the first two weeks of infection, susceptible and resistant plant cultivars have the same level of indole glucosinolates (Ludwig-Muller *et al.,* 2000). The tolerant plants increase in level of aromatic glucosinolates at 14 and 30 days after infection, susceptible plants increase in level of indole glucosinolates after 14 and 20 days. As mentioned, the primary stage of the life-cycle takes 14 days and the secondary stage is restricted to the susceptible cultivars of brassicaceae. The increase in aromatic glucosinolates instead of indole glucosinolates in the tolerant cultivars after 14 days may therefore be a defence response against the pathogen in the resistant cultivars (Ludwig-Muller *et al.,* 2000).

Factors affecting the development of clubroot disease

There are many different factors that have been identified which promote or hinder the development of clubroot. Some of these factors include soil moisture, soil temperature, pH, exudates from plant roots, calcium and soil microflora. If the influence of these factors and the interactions between them on the life-cycle of *P. brassicae* were more fully understood, these factors could be taken into consideration when developing new integrated control measures.

Factors affecting resting spore germination

Understanding the factors that influence resting spore germination could be of great importance when developing clubroot control measures as substances preventing spores

germination could be added to the soil in order to prevent disease development. If substances could be found that promoted germination, these could be added to the soil to promote zoospore release and subsequent death in the absence of a susceptible host. Zoospores lacking a cell wall are much more vulnerable to control treatments and so could be more easily and successfully targeted with treatments.

As indicated above, the germination of clubroot spores, like other spores, requires moist soil. The amount of water present in the soil may affect the length of time in which the spore germinates and high water content increases disease severity perhaps due to this fact. Germination can occur over a wide range of temperatures and pH values, however high temperatures and acidic soil are said to promote germination (Monteith, 1924). With an increase in oxygen and in the presence of optimal nutrients, the temperature for resting spore germination is lowered (Doran, 1922).

Many factors have been shown to promote resting spore germination. Kowalski and Bochow (1996) concluded that stimuli are non-specific and could come from the exudates of a range of plant roots. Ohi *et al*., (2003) reported that caffeic acid, coumalic acid and corilagin can all induce resting spore germination. Powdered seagrass, *Posidonia australis* and extracts from this plant can also stimulate the germination of resting spores of *P. brassicae* (Hata *et al*., 2002). It would appear that brief exposure to these active aromatic coumpounds are needed to activate germination of spores. Short exposures would suggest that biochemical pathways leading to germination have been switched on. A critical enzyme may be induced or activated by the removal of a germination inhibitor because of these compounds. This suggests that the spores may require specific physical or chemical treatments and/or a stimulator to germinate. This might include heat shocks or extensive washing by rain to remove endogenous inhibitors which could indicate why temperature and moisture have an effect on spore germination. Volatile stimulants activated by rain could also overcome these inhibitors. Spores do not germinate en masse and this could suggest that *P. brassicae* resting spores have specific receptor sites for germination stimulators.

Spores of *P. brassicae* may also vary in requirements for germination because of the existence of physiological races (Ellison, 1945). Spores adapted to harsh conditions may therefore germinate better than other spores in conditions that are normally considered non optimal for clubroot development (Doran, 1922). For example, spores from Brittany are more tolerant to high temperatures (Manzares-Dauleux *et al.,* 2001) and Colhoun (1953) has suggested that races of spores more tolerant to high pH exist. If there is a departure from the optimum conditions for germination of a particular *P. brassicae* race during the germination process it would be likely that the spore would be killed. This could be an important consideration in the development of clubroot control measures.

MacFarlane (1970) and Suzucki (1992) found that resting spores derived from mature or rotting galls had a higher germination rate than those derived from young galls, suggesting that there is a germination factor present in mature galls (Asano *et al.,* 2000). Ellison (1945) suggested that spores germinated in considerable quantity because of some mass effect. High levels of spores seemed to stimulate each other to germinate. Yano *et al.* (1991) established that Ca_2^+ ions from germinating spores induces the germination of surrounding spores. Therefore, host plant exudates could stimulate resting spore germination which in turn could release Ca_2^+ encouraging further germination.

MacFarlane (1970) noticed that microbial contaminants hindered germination probably due to a fungistasis effect. Asano *et al.,* (2000) also found that surface disinfected root spores showed higher germination than non-disinfected. This contradicts Chupp (1917) and Ellison (1945) who found that resting spores germinate better in non-distilled water suggesting that microorganisms could promote resting spore germination (by producing compounds that enhance the induction of germination or by degrading the pore in the spore cell wall allowing the exit of the zoospores into the soil (Moxham, 1983)). If microorganisms are discovered that specifically promote or hinder spore germination, these organisms could be added to the soil to act as a clubroot control measure.

Soil types and their effect on clubroot development

Disease severity differs with soil types and characteristics (Murakami *et al.,* 2002). Soils with high organic matter appear to favour infection (Murakami *et al.,* 2002). This may be because such soils contain more water, are generally acidic or promote soil microflora activity (Murakami *et al.,* 2002). All of these factors are thought to increase clubroot severity. Humic soils and alluvial soils with high clay content also promote disease (Murakami *et al.,* 2000). High humic soils have low drainage ability and low gas diffusity. In soils with high humic content, carbon dioxide concentration is high, especially when the temperature is raised and plant growth is vigorous. High levels of carbon dioxide increase clubroot disease (Murakami *et al.,* 2000). It also causes soil water to decrease in pH, and low pH promotes clubroot.

The rooting depths of plants in humic soils tend to be shallow and this may adversely affect plant growth and nutrient uptake, in turn promoting disease (Murakami *et al.,* 2004). Humic acids also chelate positively charged ions such as magnesium and calcium which prevents precipitation and has a positive influence on their uptake (Bingman, 1996). Humic acids also acidify the soil. Clay soils also vary in their ability to maintain nutrients and an alteration in soil nutrient balance and therefore host nutrition may affect the development of clubroot.

Sandy soils and soils with low humic content are suppressive to disease (Murakami *et al.,* 2000). Disease is negligible in low humic soil even at high moisture so the suppressive character of the low humic soil is attributed to the physical and chemical properties of the soil surface rather than high drainage capacity and high gas diffusity (Murakami *et al.,* 2004). Low humic soil is suppressive even when it has been sterilised indicating that there is no evidence for a biologically suppressive factor in this type of soil. In Tsumagoi, coral rock has been applied to soil to control clubroot. Control may be occurring due to the absorption of the spores onto the coral rock. A mixture of vermiculite in the soil also reduced disease, perhaps due to the absorption of spores onto its surface (Murakami *et al.,* 2000).

Water content of soil and its effect on clubroot

High water levels in soils generally favour disease but such levels may also lead to an increase in above ground yield indicating amelioration of the symptoms despite high levels of the disease. Colhoun, (1953) observed that high moisture in the soil, even after liming encouraged disease levels in both naturally or artificially inoculated soil. Intermediate moisture levels appears to be more critical for the development of disease symptoms, but the water level that is necessary for infection to occur varies with soil type. In organic soils, disease can occur at 60% moisture. In mineral soil, disease can occur at 9% moisture (Hamilton and Crete, 1978). As the moisture level in both organic and mineral soils increase, so does disease incidence. A moisture level of 80% of the water holding capacity of soil is generally agreed as the optimum level for disease development (Thuma *et al.,* 1983).

Excessive water could promote disease by providing more water-filled pores for resting spore germination and for zoospore movement. The moisture requirement for the germination of spores is not uniform for all *P. brassicae* spores (Doran, 1922) and this may show why high water content promotes higher disease incidence because a higher level of spores may be able to germinate if there is more moisture present. Root hair infection can occur at lower soil matric potential than cortical infection and this might reflect the need for the larger secondary zoospores (which are suggested to be released back into the soil before re-infection) to have larger water-filled pores for movement (Thuma *et al.,* 1983). Therefore, higher water levels may promote the clubbing stage of the life-cycle.

High water content in soil could also volatilise or solubilise germination-stimulating factors promoting entrance to spore membranes (Doran, 1922). Water could cause volatiles to be absorbed or released from soil particles and plant debris (French, 1992). Larson and Walker (1944) carried out a glasshouse experiment that demonstrated that in soils treated with calcium hydroxide, complete inhibition of infection at all moisture levels was seen whether the level was constantly high or constantly low but if a low

water level was fluctuated throughout the duration of the experiment, disease was promoted. In the field, moisture fluctuations could provide one explanation as to why the control of clubroot by liming in the glasshouse always shows a greater level of consistency than seen in the field. Fluctuating moisture levels may mobilize volatiles or minor elements that were suppressed when lime was added to the soil. These elements may help to promote disease by affecting either the pathogen or the host.

Fluctuating levels of moisture has also been shown to promote disease in the closely related pathogen *Spongospora subterranea* (powdery scab of potatoes) (van de Graaf *et al.,* 2007). Short pulses of water can re-vitalise volatiles (French, 1992) and may enhance clubroot due to constantly stimulating more spores to germinate and infect the host.

Resting spores can remain viable despite being repeatedly wetted and dried (Ledingham, 1939) but plants become stressed under such conditions. Therefore, fluctuating moisture levels could also affect plant roots, making them more susceptible to infection by *P. brassicae*. The oxygen demand of growing roots is high and roots can be easily damaged under poorly drained conditions. This would mean that a high water level would also damage roots making them more susceptible to disease. High soil moisture levels also stimulate the growth of secondary roots however (Larson and Walker, 1944). This may encourage more infection to take place as *P. brassicae* infects actively growing roots (Asano *et al.,* 2000). Constant saturation of soil can lead to macro and micronutrient deficiencies. Elements such as calcium are transported via waterflow and deficiency symptoms can develop rapidly if the water level is low and root growth becomes severely reduced (Marschner, 1995). The inactivity of root systems under saturated conditions can also lead to the inefficient uptake of iron, phosphorous, nitrogen, potassium and sulphur (Marschner, 1995). Reducing soil moisture levels may prevent root hair infection and this knowledge could be useful in disease control.

Temperature affects on disease development

Temperature is known to play a role in the development of clubroot (Monteith, 1924; Colhoun, 1953; Thuma *et al.,* 1983). A temperature of at least 16-21^{o}C is required for resting spore germination in the presence of a host plant (Chupp, 1917). Failure to obtain root hair infection below 18 ^{o}C has generally been substantiated. The optimum temperature for disease development is between 20-24 ^{o}C (Colhoun, 1953). *P. brassicae* spores from Brittany have been shown to be more tolerant of high temperatures than UK spores (they are effective at infecting root hairs at higher temperatures than UK pathotypes) suggesting that spores can vary in their ability to germinate at different temperatures (Voorrips, 1995). Temperature primarily affects the number of plants that become infected and higher temperatures causes higher infection rates (Larson and Walker, 1944). Temperature may also affect plant respiration rate. A high temperature causing an increased respiration rate will encourage the roots to absorb more water and nutrients from the soil which may have an effect on clubroot infection. Increased plant growth would also provide more tissue for the pathogen to infect and more photosynthates produced for the pathogen to utilise in development. Temperature can also play a role in the induction of nutrient deficiencies. For example, low temperature can affect the uptake of phosphorous (Gibson *et al.,* 2001). So while temperature affects disease incidence it does not appear to have any independent effects on clubroot severity. Instead, disease severity tends to show a general seasonal trend which follows increases in light and temperature values. So temperature and light in synergy may affect severity.

Colhoun, (1953) demonstrated that temperature had a greater effect at enhancing clubroot disease in alkaline rather than acidic soils but no possible explanation was given for this effect. Larson and Walker (1944) also mentioned that seasonal and soil factors influenced greatly the action of liming materials as clubroot inhibitors. These effects may be related to an increase in root growth in high alkaline soil at high temperature.

Light and seasonal effects on clubroot development

Less severe attacks of clubroot occur in glasshouse experiments during the winter than in summer (Colhoun, 1961). In the field, fewer plants develop disease between October and March. An explanation for these observations may be that higher temperatures and light intensity in summer months promote disease. Within a wide range of inoculum levels, the severity of disease is lowest at the lowest light intensity (Colhoun, 1961). At high inoculum levels, the number of diseased plants is not influenced by light intensity but at low inoculum levels, the most diseased plants are associated with the highest light intensity (Colhoun, 1961). Light does not influence the percentage incidence of diseased plants but it affects the severity of gall development. Clubroot severity is most influenced by light in the second and third weeks after inoculation which is when the switch between the primary stage of the life-cycle of *P. brassicae* to the secondary stage is occurring (Buczaki *et al.,* 1975). The type of light source has also been shown to be important in clubroot development. Sodium lamps result in more disease than mercury lamps and light of wavelengths 400 – 700 nm are needed for gall development (Buczaki *et al.,* 1975). It is unlikely that light directly affects the pathogen in the soil. In fact, Chupp (1917) and Honig (1931) have suggested that germination may be inhibited by light. Therefore it could be suggested that host factors affected by light in the second and third week after infection are facilitating gall development. Plants grown under conditions of high light might be expected to show greater concentrations of glucobrassicin which is an important precursor of indoleacetonitrile which plays a role in the production of clubbed tissue (Ludwig-Muller *et al.,* 1993). Higher disease indicies in plants grown in high light conditions may be a result of a higher growth rate and the availability of more photosynthates for the pathogen to utilise. Experiments carried out in Chapter 3 demonstrated that plants with larger galls had a higher fresh weight than plants with smaller galls. Light intensity could also influence root diffusates, which in turn may have an effect on spore germination and infection (Colhoun, 1961).

Glucosinolates affecting *P. brassicae*

Plasmodiophora brassicae infects plants which are members of the cruciferae. The family has a major line of chemical defence against pathogens, insects and herbivores, the glucosinolates (Verheilig *et al.,* 2000). Glucosinolates are constitutive, inactive precursors of secondary metabolites found in the outer walls and are activated in response to tissue damage/pathogen attack (Osbourn, 1996). Glucosinolates are sulphur-containing glucosides and over 100 individual glucosinolates have now been identified (Osbourn, 1996). These compounds are hydrolised by the enzyme myrosinae to yield volatile isothiocyanates (mustard oils) when plant tissues are damaged. The break down product of the glucosinolate depends on factors such as the structure of the glucosinolate, the form of myrosinase, plant species, pH, temperature, metal ion concentration in the soil and protein co-factors (Osbourn, 1996).

Plants of the genus Brassica have allylglucosinolate (sinigrin, also known as potassium myronate) as a major glucosinolate component (Feeny, 1977). This breaks down to allylisothiocyanate. Isothiocyanates are volatile, strongly phytotoxic and play a role as allelopathic agents, inhibiting the germination and growth of competing plants. Isothiocyanates are also known to be powerful antibiotics and also inhibit the growth of fungi and insects. High levels of glucosinolates are also toxic to mammals. Allylisothiocyanate can be present in dead plant tissue and can be leached from the tissue into soil by rain (Feeny, 1977). Because glucosinolates have a broad biocidal activity and are toxic to organisms, it can be presumed that microbial species present naturally on crucifers, or which can infect crucifers successfully (such as *P. brassicae*), have adapted to detoxify glucosinolates or avoid their harmful effects.

Feeny (1977) discovered that the growth rate of the cabbage aphid *Brevicoryne brassicae* was positively related with the total allylglucosinolate content of the crucifer plant it was feeding on. Discovery of crucifers by several enemy species is also aided by behavioural responses to glucosinolates or their breakdown products. It has been shown that populations of crucifer-adapted specialists such as *B. brassicae* and *Phyllotreta*

cruciferae reach higher densities in plants grown in monoculture rather in diverse vegetation (Feeny, 1977). In the wild, the diversity of the surrounding vegetation may reduce the risk of discovery of the plants by the pathogens responding to glucosinolates that they release into the soil (Feeny, 1977). *Plasmodiophora brassicae* spores are said to be stimulated to germinate by the presence of allylisothiocyanate (Hooker et al., 1945). This shows that leakage of such compounds, even in small amounts could be a liability to crucifers because it increases their apparancy to adapted enemies. This offers the possibility that treatments which reduce the leakage of these compounds could work well at providing clubroot control. Treatments that strengthen the cell wall such as high pH and high calcium could be acting to control clubroot in this way.

Plasmodiophora brassicae has been found in the root cortex of non-brassica species such as *Tropaeolum majus* and *Beta vulgaris*, where it can complete its life-cycle, and also in *Carica papaya* and *Reseda alba* where it carries out an incomplete life-cycle (Ludwig-Muller *et al.,* 1999). These species also contain glucosinolates and it is thought that clubroot symptom development might be correlated with glucosinolate content. Hypertrophy is only found in cultivars with high levels of glucosinolates, especially indole glucosinolates (Ludwig-Muller *et al.,* 1999). The absence of these compounds does not confer resistance but prevents club development.

Butcher *et al.,* 1974 showed a possible relationship between enhanced auxin levels in infected roots and indole glucosinolate degradation and suggested that host genotypes with lower concentrations of indole glucosinolates might show reduced symptoms because of a reduction in auxin. Ludwig-Muller *et al.,* 1997 observed that the total glucosinolate content in roots of susceptible plants were higher than in resistant plants.

Ludwig-Muller *et al.* (1997) also demonstrated that jasmonic acid (JA) and salicylic acid (SA) were active in the induction of glucosinolates. Plants treated with SA for two and six weeks after infection with *P. brassicae* showed a reduction rate of 50 and 80% disease index respectively. This contradicted Bodnaryk (1994) who concluded that

substances that influenced wound disease or stress induced responses in plants e.g. abscisic acid, indoleacetic acid, cytokinins, SA, sucrose, calcium, the chelating agent ethylene glycol tetraacetic acid (EGTA), chitin, chitosan and giberellic acids had no detectable effect on indole glucosinolates in oilseed rape and mustard. Therefore, the mechanism of increase in indole glucosinolates in *P. brassicae* infection is still not well understood but evidence does show that glucosinolates are important in the infection and symptom development of clubroot.

Nutrients and their effect on clubroot

A balance of essential nutrients in the soil is needed for the normal healthy growth of a plant and also for use in defence mechanisms. Experiments involving *P. brassicae* have shown that when the spore load is high in the soil, the incidence of diseased plants is not influenced by nutrition (Colhoun, 1961), specifically nitrogen, potassium or phosphorous. When the spore load is low however, nitrogen had a significant effect at controlling disease (Colhoun, 1961) and plants well supplied with nitrogen had a lower disease index than those not receiving nitrogen at a level which allowed only very slow growth. Walker and Hooker (1945) found that although a deficiency of nitrogen or potassium increased the disease index of plants in water culture, a similar effect was brought about by the provision of an excess of either of these elements. This also agrees with the conclusions reached by Pryor (1940), who found that in experiments with sand cultures, more clubbed plants of susceptible hosts occurred in the presence of an abundance of nitrogen or potassium or when nitrogen was deficient (Colhoun, 1961). Nitrogen does not influence the incidence of plants that are infected but affects the disease severity indicating that the nitrogen may be affecting or preventing the secondary stage of the life-cycle. Nitrogen may be having a positive effect on plant growth which would enable the plant to resist infection by being more healthy and able to produce more defence compounds. An excess of nitrogen, however, may be encouraging increased growth which would in turn create more tissue for the pathogen to infect. When certain elements are provided in excess to plants, uptake of other nutrients may be hindered. For example, the uptake of nitrogen antagonises the uptake

of potassium. Therefore, the increase of nitrogen preventing clubroot may be due to the effect it has on potassium.

Palm (1958) found that initial infection and development of clubs were stimulated by increases of potassium in nutrient solutions. This may be due to the fact that potassium increases root hair growth and *P. brassicae* infects growing root hairs (Asano *et al.,* 2000). In some experiments phosphorous significantly increased the disease index, whereas in others it brought about a reduction (Colhoun, 1961). Generally when the spore load is low, phosphorous level can effect control (Colhoun, 1961). Sodium, barium and magnesium also have controlling effects on clubroot (Samuel and Garret, 1944) and it is thought that this effect is a consequence of action on soil pH.

Boron has also been shown to have a controlling effect on clubroot, perhaps via the strengthening of cell walls (Webster and Dixon, 1988). It may have an indirect effect on host carbohydrate metabolism by inhibiting the conversion of sugars to starch by starch phosphorylase. Therefore its effects could interfere with starch accumulation and its utilisation by *P. brassicae* in cortical cells (Webster and Dixon, 1988). The change in pathogen development from plasmodium to sporangium in the root hairs is also inhibited by boron and boron possibly induces auxin which is involved in clubroot development (Webster and Dixon, 1988).

Calcium

The most influential nutrient at controlling disease is possibly calcium. High levels of calcium have been shown to reduce clubroot symptoms (Campbell *et al.,* 1985; Webster and Dixon, 1991 and Donald *et al.,* 2004). Myers and Campbell (1985) showed that calcium and hydrogen ions have low toxicity to clubroot resting spores. When resting spores were incubated in 1 M calcium chloride at pH 6.2 – 7.2 for one week, the infectivity of the spores reduced slightly from sodium chloride or buffer controls. The pH at which spores were incubated had no significant effect on infectivity.

Although calcium may not be affecting resting spore viability to a great extent, the calcium ion does seem to be able to suppress the development of the disease. Myers and Campbell (1985) used organic buffers to stabilise the pH independently of cation concentration and showed that as calcium increased, root hair infections and symptom development were inhibited. In another experiment, soil with a pH of 4.13 had a disease index of 41.7%. This was reduced to 14.1% without change in soil pH when calcium sulphate was added to the soil (Hamilton & Crete, 1978).

As with pH, calcium has the greatest effect on symptom development at low inoculum pressures (calcium ions can suppress the germination of resting spores if the inoculum level is low) (Murakami *et al.,* 2002). But calcium was also shown to inhibit sporangial dehiscence at high inoculum concentrations (Webster and Dixon, 1991). Webster and Dixon (1991) demonstrated that if calcium was applied at day 0, root hair infection was reduced. If it was applied within seven days of inoculation, the dehiscence of mature sporangia was suppressed. If the calcium concentration was raised seven days after plants were inoculated with *P. brassicae*, the course of pathogenesis was unaffected. Webster and Dixon (1991) therefore suggested that the regulation of pathogen growth in root hairs by calcium operates at fixed points in time rather than at specific life-cycle stages. Webster and Dixon (1991) also suggested that inhibition of the life-cycle of *P. brassicae* may result from calcium accumulation until a suppressive level is reached. This was because low calcium concentrations required application over a longer period of time than higher applications to achieve similar reductions in pathogen development. No direct relationship between host calcium content and symptom reduction has been demonstrated, but the incorporation of calcium into uninoculated roots is depressed at pH 6.2 compared with pH 6.8 or 7.2 (Myers and Campbell, 1985). This may indicate why high pH and calcium together are more effective at controlling *P. brassicae* than when used individually. Calcium is involved in another type of mineral antagonism. It is involved in the potassium-calcium-magnesium interaction. Any one of these elements in excess can cause a decrease in the uptake of the other. These results show that the

balance of nutrients in the soil greatly affects clubroot and this needs to be taken into account when developing control measures.

pH

Clubroot symptoms can occur at pH 4.1-8.8 (Colhoun, 1953) but disease incidence generally decreases at high pH (Murakami *et al.,* 2002). Poor uptake of nutrients, particularly boron, copper, manganese and zinc can occur if the pH of the soil is above 6.5 so pH may exert an effect on clubroot disease through altered nutrient uptake by the plant. pH is one of the most influential factors on clubroot disease. The optimum pH for root hair infection is pH 4.5 – 5.2 (Palm, 1963) however root hair infection can still occur at pH 7.9. Root hair infections and their rate of maturation are reduced sharply at pH values higher than 7.2 (Webster & Dixon, 1991). This is thought to be a result of abortion of primary thalli before release of secondary zoospores (Myers and Campbell, 1985). The optimum pH for galling to occur is pH 5.9 (Palm, 1963) but galling generally fails above pH 7.5. Nevertheless, good clubroot control can be achieved even if the critical pH of 7.2 is never reached (Tremblay *et al.,* 2005). Soil pH has the greatest effect on symptom development at low inoculum pressures and it is said that for good control, a high pH for a long period of time is needed (Colhoun, 1953). It has also been observed that it is less common to see heavy clubroot attacks in alkaline soil in pots than in the field and an uneven distribution of soil acidity in the field may account for this (Colhoun, 1953).

Hydrogen ions are involved in the maintenance of charge balances and pH regulation of parasitic associations with plants (Smith and Smith, 1990). High pH could therefore directly affect *P. brassicae*. Membranes become depolarized due to a change in pH and become more permeable (Naitoh *et al.,* 1972). *P. brassicae* does not have ATPase which is an enzyme that imports many metabolites necessary for metabolism, exports toxins, wastes and solutes, and also establishes the ionic balance that maintains the cell potential. Therefore, if the pH of the soil was drastically altered, *P. brassicae* may be unable to regulate the pH balance and would become vulnerable to unwanted apoplastic

solutes such as calcium and sodium (Smith and Smith, 1990). At pH values above 6.5, cells also become permeable to fatty acids which have been shown to be toxic to fungi (van Rijn and Termorshuizen, 2007). It has been shown that extracting resting spores in buffers at pH 9.8 does not affect the viability of the spores (Castlebury *et al.,* 1994) and spores are able to germinate at pH 8 (Ellison, 1945). This may mean that pH might not have an effect on resting spore germination but may have an effect on the development of *P. brassicae* in the roots by causing the primary thalli to abort.

The effect of pH on clubroot control may also be due to an effect on the plant host or soil microflora rather than on the pathogen. Soils with different pH values contain different microorganisms that are more tolerant to those pH values (Hashiba and Narisawa, 2005; Chang and Lynd, 1970). Certain fungi grow after the addition of lime (Buscot and Varma, 2005). Soil pH affects the level of fungi in the soil by affecting the solubility of several compounds thereby influencing different microorganisms that have different nutritional requirements (Buscot and Varma, 2005). Alteration to the soil microflora could affect *P. brassicae* as microorganisms present in high pH soils may be antagonistic or produce compounds that are toxic (Mallik, 2001).

Low cell wall pH is also necessary for wall loosening in plants and for promoting the antagonism of auxin. Therefore, the alteration of soil pH could interfere with the primary and secondary invasions and the cortical migration and host cell hypertrophy in clubroot development (Dixon and Webster, 1988).

The pH of the soil may also affect auxin levels directly. At an alkaline pH, auxin is in a form which is less permeable than under acidic conditions (Schopfer, 1993). Auxin increases the frequency of lateral root formation and auxin response sites for this effect are behind a permeation barrier. Therefore, the inhibition of lateral root formation might restrict the available root volume which is susceptible to primary invasion and so may account for the decreased infections at increased pH (Schopfer, 1993).

High soil pH may also alter host nutrition. High pH mobilises minor elements such as iron and copper and other elements that are essential for good plant growth and the production of secondary metabolites that are needed for plant defense responses (Huber, 1990). It has also been shown that plants that are resistant to *P. brassicae* cause an alkalization of the root zone (Takahashi *et al.,* 2006; Fuchs and Sacristan, 1996). This alkalization is regulated by H^+ATPase and would suggest that H^+ATPase plays a part in the plant defence against clubroot (Takahashi *et al.,* 2006). By altering the pH in the root zone, the plants may be making conditions unfavourable for pathogen invasion. For example, *Alternaria solani* counters the effect of saponins by lowering the pH at the infection site to levels at which the saponins are ineffective (Osbourn, 1996). *P. brassicae* may have similar mechanisms to overcome plant defences and resistant plant hosts may alkalise the root zone to counteract this effect. Rapid alkalisation also occurs in the medium of *Lycopersicon pelvianum* cells in response to systemin which mediates wound-induced defence responses. The alkalisation was suppressed by fusicoccin which is an activator of plasma membrane H^+ATPase which showed that medium alkalisation was involved in the influx of H^+ by H^+ATPase in response to wounding (Takahashi *et al.,* 2006). Clubroot resistant plant cultivars might also specifically recognise *P. brassicae* as an elicitor and induce defence responses accordingly by alkalisation of the root zone by regulation of H^+ by ion pumps like H^+ATPase. The addition of lime to alter the soil pH could be activating H^+ATPase which would help in the prevention of disease. Lime could be mimicking the defence response of resistant plants. Takahashi *et al.,* (2006) showed that defense against clubroot was resistance specific as there were no changes in the pH of the soil and cell viability in susceptible roots.

Spore load/inoculum potential

No relationship has been found between the percentage of root hairs infected and spore concentration (Takahashi *et al.,* 2006; Voorrips, 1995). The infection efficiency of spores is about 3-4% of the total root hairs present on a root system. This is the same regardless of whether the roots are infected with a low amount or high amount of spores (10^4-10^6 spores/g soil) (Takahashi *et al.,* 2006). This would suggest that the infection

rate in root hairs is saturated upon exposure to 10^4 spores/g soil. There is an increase in disease severity with an increasing level of spores in the soil but a low survival of spores in the root hairs can also lead to the infection of the cortex (Voorrips, 1995). Murakami, *et al*., (2002) showed that dose response curves of *P. brassicae* and their effect on clubroot severity varied even when soils were of similar pedological type. Jones and Ingram (1982) saw that pathogenic isolates can be inhibited by non-pathogenic isolates. There is also evidence that large amounts of spores can stimulate the germination of other spores (Wellman, 1930; Yano *et al.,* 1991) and this might explain the differences in disease severity caused by spore loads of the same amounts. In general, disease severity is not consistently related to disease incidence but higher amounts of spores in the soil do lead to higher disease severity.

The effect of treatments aimed at controlling disease can also vary with the number of spores present in the soil, for example, lime and nutrients such as nitrogen can control clubroot but only if the spore load is low (Colhoun, 1953; Hamilton and Crete, 1978). The effect of the spore load on the number of diseased plants is also much greater in alkaline than in acid soils (Colhoun, 1953). Calcium can also suppress the germination of resting spores in the soil if they are at a low density (Murakami *et al.,* 2002). The rate of development of *P. brassicae* in root hairs also varies with inoculum pressure. If the numbers of spores in the root are reduced they are more susceptible to control by the plant and high pH and calcium may help the plant in its defence response against the organism. Therefore, the knowledge of the spore level in soil may assist in recommendations of various control treatments.

Microorganisms

The presence of microorganisms on resting spores can either hinder or promote germination. Certain microorganisms may produce compounds that are fungistatic or fungitoxic to the spores. The middle layer of the cell wall of *P. brassicae* contains chitin and the thickening of the middle wall layer is known to be susceptible to chitinolytic enzymes (Tanaka *et al.,* 2001). Therefore, chitinolytic organisms may enhance resting spore germination by being involved in the formation of the opened germ pore in the

resting sporangium of *P. brassicae*. The cell wall also contains a high level of protein. Buczacki (1983) showed that there were enhanced root hair infections when root hairs were suspended in non-sterile soil and the factor responsible for this enhanced germination was found to be a protein that was induced by other soil microorganisms. Its affects were mimicked by treating spores with proteolytic enzymes. This would suggest that an increase in infection may have been due to the breakdown of proteins localised in the outer wall of the resting spores by certain microorganisms.

High levels of microorganisms in the soil may also compete with *P. brassicae* spores for resources and may reduce the effectiveness of infection of the pathogen. In addition, high levels of microorganisms in the soil may also provide compounds that are beneficial to the plant host promoting growth and potential to deal with infection. The influence of soil microflora on clubroot development is therefore complex and so the presence of different types of microorganisms present in field soil may account for differences in disease levels observed in soils of the same type in different locations.

The root endophytic fungus *Heteroconium chaetospira* has been isolated from roots of Chinese cabbage in Japan (Hashiba and Narisawa, 2005). This fungus is an effective biocontrol agent against clubroot in Chinese cabbage at low to moderate soil moisture range and a pathogen resting spore density of 10^5 spores/g soil. The fungus penetrates the outer epidermal cells without any degradation, and three weeks after infection, hyphae are abundant in the epidermis and outer cortical layer including those of the tip region of the roots. There are few hyphae in the inner cortical layer and none in the vascular cylinder. The organism exchanges nitrogen in return for carbon. It persists in the roots and induces systemic resistance to foliar disease. The biocontrol potential of this organism may be a product of competition for physical space or as a result of stimulation of host defence (Hashiba and Narisawa, 2005). In addition, the nitrogen supplied to the plant by this organism may also be a factor in the control of clubroot disease by this organism. Commercial horticultural soils containing excess nutrients are not suitable for *H. chaetospira* growth in Chinese cabbage indicating that there may be

an interaction between nutrients and microorganisms that can affect clubroot development (Hashiba and Narisawa, 2005). The types of organisms that promote or hinder clubroot development and the interaction they have with their environment have not been studied in detail.

Pathogen virulence

Different races of *P. brassicae* exist and this is discussed further in Chapter 2. Certain spore races differentially infect different plant hosts. Therefore, the plant species that are planted in a particular soil will have an effect on the disease level that can be observed. The virulence of the pathogen also affects disease severity and some races of the pathogen are more virulent than others. For example, mature spores are more able to infect hosts than immature spores (Suzuki *et al.,* 1992). Some spores may also be better adapted to various conditions such as temperature and pH which will in turn affect disease level in different conditions. Knowledge of the particular pathotypes of spores within a field would therefore be useful in the development of clubroot control measures because soil conditions could be altered to make them more detrimental to the specific pathotype present in the soil, or plant cultivars with specific resistance to a certain field spore population could be used in that location.

Plant genotype

Disease development is affected by host susceptibility. Some plants or cultivars may have high levels of resistance to *P. brassicae* such as the plants tested by Takahashi *et al.*, (2006) that produced a hypersensitive response to infection and prevented the development of clubbing symptoms. Fuchs and Sacristan (1996) also observed a hypersensitive response (HR) from *Arabidopsis thaliana* when infected by *P. brassicae.* Other plants or cultivars may have low levels of resistance which are effective at low levels of inoculum densities (e.g. oilseed rape from Sweden which are resistant if the spore load in the soil is low but not if it is high (Wallenhammar, 1996)). Plant cultivars may have differential resistance towards the various races of *P. brassicae*, self evident from the use of different genotypes to assign the race of spore pathotypes – the European Clubroot Differential (ECD) set. This is described in more detail in Chapter 2. Some of

the genes involved in plant resistance against *P. brassicae* have been identified. In Chinese cabbage (*B. campestris* ssp *pekinesis*), CRb has been found to be a dominant gene for resistance (Piao *et al.,* 2004) providing evidence that control against clubroot is related at least partially to the gene-for-gene theory in this example. In *B. oleracea*, nine genomic areas have been detected as inferring resistance (Rocherieux *et al.,* 2004). Single genes or quantitative trait loci (QTLs) which involve multiple genes are therefore involved in the resistance of *B. oleracea* towards *P. brassicae*. These resistance areas could be bred into commercial cultivars and a combination of single genes and QTLs may provide a broad-spectrum and durable strategy for the control of clubroot. The use of resistant cultivars would also potentially reduce the spore inoculum in the soil because *P. brassicae* only completes the root hair stage of the life-cycle in resistant plants and therefore new resting spores would not be returned to the soil.

Current control measures

As a result of current knowledge of the life-cycle of *P. brassicae* and the factors that affect the development of the disease, existing control measures for clubroot focus on crop rotation to try and reduce the build-up of spore inoculum in the soil. The length of the rotation in theory is a function of the long life span of *P. brassicae*. Growers prefer narrower rotations however, or even continuous cropping to maximise profits. Also, the presence of cruciferous weeds in the field can harbour clubroot spores and so inoculum may still be able to be produced in the soil despite the absence of a brassica crop.

Early workers associated clubroot disease with lime deficiency. Therefore, lime has historically been recommended and applied for clubroot control but it is not always effective (Dobson *et al.,* 1983). Different liming materials not only affect the soil pH but can affect the exchangeable calcium content and other nutrient levels in the soil to different extents. Calcium in the soil breaks up clay particles forming a granular structure, which aids in the aeration of soil. Improving soil texture improves soil drainage, and liming also encourages the activity of earthworms and micro-organisms in the soil (Buscot and Varma, 2005). Despite all of these effects, an increase in soil pH and cation concentration are the two main factors influenced by liming that are thought

to affect clubroot disease control (Campbell *et al.,* 1985). Liming of soil to produce a pH of 7.2 is currently aimed for because this pH is said to be the optimum pH for disease control (Campbell *et al.,* 1985). However, Colhoun (1953) established that disease can occur in both acid and alkaline soils. Disease indices are also different in soils with the same pH (Murakami *et al.,* 2002).

The fact that lime does not give consistently good control could be due to soil factors influencing the effectiveness of lime. The differences in the capability of lime to control clubroot disease may be related to soil type. This may reflect the efficacy of lime on altering measured pH among soil types. In a glasshouse experiment carried out by Myers *et al.,* (1981), limed autoclaved soils were inoculated with 10^4 resting spores/g soil and sown with broccoli. Lime at 2.8, 5.6, 11.2, and 22.4 tonnes/ha controlled clubroot in ten of the soils termed 'lime responsive.' There was virtually no disease with the lowest rate of lime treatment at a soil pH as low as pH 6.7. However, lime gave poor control in four soils termed 'lime non-responsive' with lime treatments as high as 11.2 t/ha and a soil pH as high as pH 7.7. The factors influencing lime-responsiveness are unknown, but this again demonstrates that soil pH alone is not a reliable indication of the potential for clubroot control in all soils. Campbell *et al.,* (1985) also indicated that cations may play a smaller role in clubroot control than pH, but their relative importance may differ in the complex environment of field soils. In different soil types the effectiveness of lime will depend on a number of factors including the buffering capacity of the soil, the organic matter content and the ratio of nutrients in the soil (Donald *et al.,* 2006).

Some soil textures may enable lime to be better distributed or re-distributed than others (Dobson *et al.,* 1983). Less calcium is needed in sandy soils than in silty, clayey and organic soils for optimal plant growth (Kelling and Schulte, 2004). This may indicate why there is generally better clubroot control in sandy soils. Sandy or coarse-textured soils have a low cation exchange capacity, whereas medium or fine-textured soils have a higher cation exchange capacity (Schulte and Kelling, 1993). The inherent differences

among soils in their available cations could therefore account for the reported failure of liming to control clubroot (Campbell *et al.,* 1985). Calcium requirement for optimal plant growth increases with increasing external concentrations of heavy metals due to its replacement with other metals at binding sites. The mineralogy of the soil is therefore also an important factor in determining optimum percent saturation of exchange sites with a given cation (Schulte and Kelling, 1993) and hence clubroot control. The soil microflora could also have an effect on the effectiveness of lime as a clubroot control measure. All of these differences in soil types have to be taken into account when developing control measures for clubroot so that the treatments will have an optimal effect at controlling disease across many different types of soil

The fertiliser calcium cyanamide has been used for over 100 years in clubroot control but again its effectiveness may be influenced by other factors (Williamson and Dyce, 1989) and in addition can often be phytotoxic (Williamson and Dyce, 1989). Adding nutrients such as boron to the soil is also used as a control measure but again, this has not provided adequate control.

Resistant cultivars are the most effective way of reducing disease caused by *P. brassicae*. Few resistant plant cultivars are available as natural resistance from wild plants is not common or easily transferred into commercial crops, and the resistant cultivars may only be resistant to certain pathotypes of the pathogen. There is also a danger that races could develop that may overcome the genetic resistance of the cultivars.

These existing control strategies, each of which may be only partially effective, may still have a role to play in controlling clubroot, however, growers require new cost effective and sustainable solutions so that crop losses can be reduced and vegetable production can be sustained.

New and existing control measures

Substances that inhibit resting spore germination

The germination of the resting spore is often seen as the first critical stage in the development of clubroot disease. Therefore, treatments that can kill the spores or prevent the spores from germinating would be valuable as a control measure. Specific fungicides targeting the resting spore would be the most effective treatments. The fungicides cyazofamid and fluazinam have been shown to have an effect at preventing clubroot disease (Mitani *et al.,* 2003; Donald *et al.,* 2006). One project aim was to carry out field trials under commercial conditions to determine if these fungicides would be able to be used as a practical clubroot control measure in the UK as fluazinam had been observed to be effective in Australian field trials (Donald *et al.,* 2006) and cyazofamid had been observed to be effective against *P. brassicae* spores in the glasshouse (Mitani *et al.,* 2003).

With increasing concern over the use of all agro chemicals throughout Europe and wider afield, non-chemical approaches to control clubroot would have potential. Microorganisms that produce compounds that are toxic to the spores could be added to the soil (Mallik, 2001). Microorganisms that produce substances that break down the spore cell wall such as chitinases and proteinases could also be added to the soil in an attempt to kill off clubroot resting spores. Therefore individual microorganisms, mixtures of microorganisms and treatments such as spent mushroom compost which contain high levels of chitinolytic organisms (Guo *et al.,* 2001) may be useful as a clubroot control measure. Also, treatments that stimulate the production of antagonistic microorganisms in the soil could be effective as a clubroot control treatment. These are completely novel treatments that have not been tested in a field environment against *P. brassicae*.

Substances that promote resting spore germination

By artificially promoting the germination of the resting spores in the soil in the absence of a host plant, the zoospores would die and would be unable to infect subsequent host transplants. Promoting premature germination could be achieved by adding germination- stimulating factors to the soil well in advance of transplanting the crops. Some germination factors that have been described are glucosinolates and calcium (Walker *et al.,* 1945; Yano *et al.,* 1991). Therefore lime treatments and oilseed rape (containing glucosinolates) could be added in advance of transplanting to promote germination to reduce the number of infected plants. In both cases it would be necessary to determine the optimum timing of addition of treatments. Decoy crops such as radish can stimulate the germination of resting spores (Murakami *et al.,* 2000) so these crops could be planted into the soil prior to the brassica crop in order to lower soil inoculum in the field.

Surfactants to destroy resting spore walls

Surfactants act by altering spore membranes by binding to their lipids. *Plasmodiophora brassicae* has high levels of lipids in its spore walls (Moxham, 1983) and so this approach may be expected to it be highly effective at destroying clubroot spores. Different types of surfactants have different effects on membrane lipids and so finding a surfactant that is specific to the *P. brassicae* cell wall would be a necessary first step to determining the potential of this methodology.

Altering nutrient level of the soil to prevent clubroot

By affecting the attachment of the zoospores onto the root hairs (in either a physical or chemical recognition sense), disease could be controlled or prevented. The nutrient balance in the soil would be likely to affect this process by altering root properties to make them less easy to penetrate or improving host nutrition so that they can deploy effective defence responses (Thomas *et al.,* 2000). Adding lime treatments that increase soil pH and calcium levels would affect root wall strength. Treatments like boron can also control clubroot and its efficacy increases when used with calcium. Therefore,

combinations of treatments that provide various nutrients to the soil could be developed to maintain a nutrient balance in the soil that is favourable to the plant host but detrimental to the pathogen.

Discovering the most effective form of lime to use in control

Lime is not a new control measure as it has been used for over a hundred years to effect control, however, it is not fully effective. Plants grown in growth chambers in mineral and organic soils have shown the lime source to have a greater effect on clubroot control than soil pH (Fletcher *et al.,* 1982). Hamilton and Crete (1978) also suggested that the reduction of clubroot was more dependent on liming source than pH. Murakami *et al.,* (2002) analysed the results of disease reduction using different types of lime and showed that there was a significant interaction between the lime materials and the effect on soil pH and the extractable calcium that they provided to the soil at reducing disease. This implied that the effectiveness of soil pH and calcium ratios on the suppression of clubroot disease may differ among different liming materials. If a particular type of lime could be shown to be consistently better at preventing disease than others, this could be of value in developing sustainable control measures. The fineness of the lime and the timing of addition of this treatment is also important in the level of control achieved and this could be investigated.

Types of lime used in clubroot control

Calcium carbonate

Calcium carbonate has been shown to reduce symptom expression when applied during primary colonization therefore, calcium carbonate affects root hair colonisation (Webster & Dixon, 1991). Calcium carbonate increases the pH and exchangeable calcium content of the soil when more is added but level of disease reduction achieved tends to stay the same despite the increase (Murakami *et al.,* 2002). (This observation was also made in the glasshouse experiments described in Chapter 3). Calcium carbonate can control disease when it causes the soil to reach pH 7.9 in the field

(Wellman, 1930) whereas Larson and Walker (1944) found that calcium carbonate controlled disease when the soil pH reached 7.2 in the glasshouse. It has also been shown that the inoculum level of the soil does not decline appreciably when calcium carbonate is applied (Fletcher *et al.,* 1982). Calcium carbonate increases root yields more significantly than any other type of lime with or without the presence of infection and the addition of calcium carbonate to the soil also increases crop yield (Fletcher *et al.,* 1982).

Calcium hydroxide

Webster and Dixon, (1991) demonstrated that calcium hydroxide reduced symptom expression when applied during primary colonization. Calcium hydroxide has different effects on clubroot in different types of soil. For example, adding calcium hydroxide to unlimed organic soil can increase disease (Hamilton & Crete, 1978). In mineral soil, calcium hydroxide increases the soil pH and exchangeable calcium content and reduces disease (Hamilton & Crete, 1978). Wellman (1930) showed that disease was controlled when soil pH was raised to 7.3 by calcium hydroxide in the field. Under glasshouse conditions, Larson and Walker, (1944) showed that it needed to raise the soil pH to 7.2 to get good control. Murakami *et al.,* (2002) showed that calcium hydroxide decreased the disease level; and the disease level kept decreasing as more was added and the pH and extractable calcium levels increased. Using calcium hydroxide can give better plant yields compared to any other type of lime whether in the presence of spore inoculum or not. It gives significantly higher yields than the yields obtained by the addition of calcium carbonate (Hamilton & Crete, 1978).

Calcium oxide

Disease index was shown to be reduced by 20 - 39% when 2g/kg of CaO was applied to clubroot inoculated soil (Hamilton & Crete, 1978) demonstrating that calcium oxide had an effect on clubroot severity. A powdered form of this treatment was demonstrated to be extremely effective at controlling clubroot in glasshouse and field trial experiments (Chapters 3 and 4).

Calcium sulphate

Calcium sulphate does not increase soil pH and may actually decrease it over time (Fletcher *et al.,* 1982). In spite of this, Fletcher *et al.,* (1982) showed that the level of disease incidence is significantly lower in clubroot infested soil amended with calcium sulphate than in untreated soil, indicating that a factor other than pH must influence the level of disease (Fletcher *et al.,* 1982). It may be that the influence of calcium sulphate on soil acidity favours clubroot development but simultaneously the calcium ion has an inhibitory effect on the disease development (Hamilton & Crete, 1978). Conversely, Donald *et al.,* (2006) showed that calcium sulphate did not control disease. This agreed with an experiment discussed in Chapter 3 in which calcium sulphate had no significant effect on clubroot. Webster and Dixon (1991) demonstrated that calcium sulphate affected *P. brassicae* during both the primary and secondary phase of development because calcium sulphate applied during either primary or secondary colonization limited club development. Calcium sulphate also increased crop yield in the presence of disease (Fletcher *et al.,* 1982). Calcium sulphate contains 20-22% calcium. It is not recommended as a source of calcium except for soils with a low cation-exchange capacity (Kelling and Schulte, 2004) and the difference in soil types used in the various experiments may explain the reason as to why there were contradictory reports about the effectiveness of calcium sulphate as a clubroot control measure.

Dolomite

Dolomite, also known as calcium magnesium carbonate is a lime that contains a high content of magnesium compared to other limestone such as calcium carbonate. An experiment by Murakami *et al.,* (2002) demonstrated that when 2 g/kg of dolomite was applied to clubroot infested soil, the pH of the soil and the exchangeable calcium content of the soil stayed the same as more was added but that the disease decreased. A reduction of around 30% was gained. Larson and Walker (1944) showed that when dolomite raised the pH of the soil to pH 7.2 in the glasshouse, disease was prevented.

Calcium nitrate

Calcium from calcium nitrate is taken up preferentially by host plants over other forms of calcium and when calcium nitrate was added to the soil along with other forms of lime the combination was consistently more effective at controlling clubroot than the individual treatments (Donald *et al.,* 2006; Dobson *et al.,* 1983). However, a glasshouse experiment discussed in Chapter 3 demonstrated that calcium nitrate had no significant effect at controlling clubroot when used on its own.

Summary on types of lime used for clubroot control

From the literature there is a consensus that lime in the carbonate or hydroxide form would be the most effective treatment for providing control. In many past experiments calcium hydroxide gave the best level of disease control and also the highest increase in yields. Calcium carbonate however is more readily available and lower in cost and if a high pH could be achieved using this treatment, it could provide a high level of control and increase plant yields. Spent lime is also readily available and would be a cost-effective form of lime to use. This project aims to discover which of these types of lime would give the most effective level of clubroot control over the widest range of environmental conditions and soil types.

Calcium cyanamide

Calcium cyanamide has been used to control clubroot for over 100 years. It is used as a slow-release fertilizer, herbicide, pesticide and fungicide. The breakdown products of calcium cyanamide are CN_2 (which is fungitoxic), calcium hydroxide and urea (Williamson and Dyce, 1989). The calcium hydroxide component raises soil pH and calcium when it is hydrolysed. Murakami *et al.,* (2002) observed that calcium cyanamide increased the pH and exchangeable calcium content in the soil as more was added which in turn caused a reduction in disease. However, experiments discussed in chapters 3 and 4 showed that calcium cyanamide did not significantly affect soil pH or calcium levels in the glasshouse or field but did reduce disease. Root hair infection in calcium cyanamide amended soils is usually slightly lower than for other types of lime

(Murakami *et al.,* 2002) and it has been shown that the germination of resting spores is inhibited by calcium cyanamide *in vivo* (Murakami *et al.,* 2002). Calcium cyanamide also increases plant yield probably due to the nitrogen content of this treatment (Williamson and Dyce, 1989), but can be phytotoxic if applied too close to planting (Murakami *et al.,* 2002) and at high levels (Williamson and Dyce, 1989). The phytotoxicity of calcium cyanamide may vary with soil type and environment (Williamson and Dyce, 1989). The phytotoxic effect of calcium cyanamide is also more evident with cultivars that are resistant to *P. brassicae* (Williamson and Dyce, 1989). The addition of calcium cyanamide has to be proportional to the level of inoculum to get control e.g. if there is high inoculum, high levels of calcium cyanamide have to be added for good control (Williamson and Dyce, 1989). Calcium cyanamide is usually added to the soil in the form of a granular product called Perlka. However, the powdered fraction of this treatment has been shown to have the greatest controlling effect on clubroot (Donald *et al.,* 2004b). This project aimed to find a level of calcium cyanamide that could effectively control disease over a wide range of conditions and to discover a method of timing and application of this product that would not be phytotoxic.

Alternatives to lime

Other treatments may add calcium and nutrients to the soil and raise soil pH and may be worth pursuing as clubroot control measures instead of the conventional lime treatments. Treatments such as shell sand (a waste product of the fishing industry) may provide these features when added to the soil.

Altering soil drainage properties to improve clubroot control

Increased soil water content increases disease so improving the drainage of the soil could also help reduce disease level. Deep ploughing and creating ridged beds could help with soil drainage. Growing brassicas on raised beds is not widely carried out on the UK but is routinely practiced by growers in Australia in the state of Victoria (Donald *et al.,* 2006). It would be useful to determine if this method is effective in disease control here.

Strategic application of treatments to the soil

Applying treatments to the soil in bands around the root balls of the transplanted crop could help improve the efficacy of treatments and reduce the cost of treatments due to the reduced quantities of materials used using this strategy. Vegetable brassicas have a relatively dense, compact root structure and the zoospores of *P. brassicae* have limited motility in the absence of excess water and therefore adding treatments specifically around the root ball instead of the usual broadcast application would be suited to these crops.

Decoy plants as a method of clubroot control

Planting crops amongst crucifers to make the commercial crucifer plants less easy to detect by the pathogen is also another strategy for control. Similarly, ryegrass has been found to prevent disease development (Murakami *et al.,* 2000). Therefore planting this sort of crop along with brassicas may reduce the disease pressure on the brassica plants but competition effects between the plants would have to be examined before this control measure could be used optimally in the field.

Minimising the effects of light and temperature on clubroot severity

Light and soil temperature affects clubroot disease development so growing brassica crops at different times of the year from normal standard practice could be a good way of minimising clubroot attacks. This has been achieved in Queensland, Australia (Donald *et al.,* 2006) but may not be a practical method of clubroot control generally due to market forces and management considerations (Donald *et al.,* 2006). Reduced light would also potentially give reduced yield.

Combinations of treatments for control

Combinations of different treatments that attack different stages of the life-cycle of *P. brassicae* could be added to the soil to provide an additive or synergistic effect on disease control. In field trials carried out in the state of Victoria, Australia, the integration of two or more treatments such as a fungicide, nitrogen fertiliser and lime, was more effective at controlling clubroot than applying any single treatment on its own

(Donald *et al.,* 2006). However, this was effective only in soils of low pH. Adding combinations of conventional treatments such as lime and fungicides with more novel approaches such as biocontrol agents, could potentially give enhanced clubroot control and increased plant yields.

Quantifying spore load in the soil

With no satisfactory control measures for clubroot, attempts have been made to develop methods of detecting and quantifying *P. brassicae* resting spore numbers in soil to enable growers to avoid fields with high levels of inoculum.

High levels of spore inoculum are not controlled by factors such as lime and nutrients, and some resistant cultivars may only be resistant to low levels of inoculum (Wallenhammar, 1996). By quantifying the level of spores present in a field, the optimum treatments could be advised for clubroot control in a field. For example, if a high spore load is present, treatments such as fungicides or calcium cyanamide or combinations of treatments could be used for control. If the spore load is low, less expensive treatments such as lime could be used. The spore load in the field could also be linked to predicted yield loss so that the treatments chosen for control would give economic returns. Therefore, the detection and quantification of *P. brassicae* in field soil is also an important tool in the development of clubroot control.

Current methods for clubroot detection in soil

Plasmodiophora brassicae cannot be cultured and therefore bait plants have been utilised as a method of establishing the presence of *P. brassicae* in soils (see Chapter 5 for a description of bait plant tests). This method can only detect inoculum levels of greater than 1000 spores/g dry soil (Wallenhammar, 1996) and this method, although reliable, is slow and expensive in terms of both time and facilities. Large numbers of samples can cause difficulties because of the space that it requires in the glasshouse to carry out, and a trained specialist is needed for root examination. Assessments are based on subjective appraisals, based on an arbitrary system of disease indices, which may be

sufficient for advisory purposes in allowing growers to avoid high risk fields but would give no indication to specific control treatments that could be added to the field to reduce this level of disease severity.

Detecting the presence of *P. brassicae* by carrying out microscopic observations on root hair infection is faster than waiting for the clubbing symptoms to develop but no linear relationship exists between root hair infection and subsequent gall development (Voorrips, 1996). Therefore this method is not useful for quantifying initial spore level in the soil and the disease level that it will cause.

Using fluorescence microscopy to detect clubroot spores directly from soil is possible (Arie *et al.,* 1988) but also requires a specialist who is trained to differentiate the spores from other fluorescent objects in the soil. The binding of spores to soil particles can also result in an under-estimation of the spore load using this technique (Faggian *et al.,* 1999). This method is therefore not practical for the development of a rapid, accurate diagnostic test.

Serological detection is also not an effective method for estimating the numbers of resting spores in the soil. This is because there are many races of *P. brassicae* and a single root gall can be composed of many races of the pathogen and changes in the composition of each pathotypes DNA may not be detectable. It is also not an effective technique because the sample preparation is very laborious (Faggian *et al.,* 1999). A dipstick assay, indirect ELISA and indirect immunofluorescence assay with a detection limit of 10^2 resting spores/g soil was developed by Wakeham and White, (1996) by raising antiserum to sonicated resting spores. The use of polyclonal antibodies in a commercial sense is limited however by the finite life of the antisera and the potential for cross-reactivity with other microorganisms found in soil such as *Sclerotium rolfsii* (Donald *et al.,* 2006).

A technique to detect the presence of *P. brassicae* in soils using nested polymerase chain reaction (PCR) has been developed by Ito *et al.,* (1999) and Faggian *et al.,* (1999). This

test is rapid, sensitive and specific and can be carried out by non-specialist personnel (Ito *et al*., 1997). This reduces testing time, labour costs, and also increases turnover. Primers specific to *P. brassicae* DNA are able to detect the pathogen is soils but there are problems with low amplification efficiency and false negative results (Donald *et al.,* 2006). Extensive purification of DNA extracted from soil is also necessary to remove substances that inhibit PCR and this has been shown to be difficult by many different researchers. Another problem with nested PCR is that this sort of test only detects the presence of *P. brassicae* DNA and does not quantify it. Therefore, a rapid, sensitive and specific test for detecting and quantifying the numbers of *P. brassicae* resting spores in the soil is still required.

The use of real-time PCR would offer an improved method to use for this test. During real-time PCR, unlike conventional PCR, the accumulation of PCR products is measured automatically after each cycle using an integrated cycler/fluorimeter. Direct measurement of the accumulated PCR product allows the phases of the reaction to be monitored. The initial amount of target DNA in the reaction can be related to a 'cycle threshold' (C_t), defined as that cycle number at which a statistically significant increase in fluorescence is detected. Target DNA can then be quantified by constructing a calibration curve that relates C_t to known amounts of template DNA (McCartney *et al.,* 2003). PCR products can be monitored using either fluorescent DNA-intercalating dyes (e.g. SYBR Green) or sequence-specific probe-based assays.

Real-time PCR primers and probes that are specific to *P. brassicae* have already been developed (Faggian *et al.,* 2003; Central Science Laboratories (CSL), Ian Barker, pers. com.) and so the development of a quantitative real-time PCR diagnostic test for the quantification of *P. brassicae* resting spores directly from soil is achievable. The major aim of developing this diagnostic test would be to relate the results given by the test to the field. Therefore, the initial levels of spore inoculum determined by the test would have to be compared to disease levels seen in the field. This is a complicated procedure because as stated above, the level of infection and the extent of pathogen invasion is partly dependent upon a range of environmental factors that can increase or decrease the

damage caused to the host by the pathogen, not just the initial spore load. These factors include pathogen virulence and pathotype, soil moisture, soil temperature, host resistance, soil pH and nutrient level and soil microflora. The results could therefore be related to validated bioassay tests to try and predict possible disease severity in the field. These validated tests would have been developed using known spore loads on different soil types in a controlled environment.

Conclusion and further aims

The development of novel control measures for clubroot and the optimisation of use of conventional control measures tested in this project will be of great benefit to brassica growers. New sustainable methods of control that give consistent disease reduction and an increase in yield are required so that brassica crop losses can be reduced and production can be sustained. It is hoped that the testing of novel control treatments against clubroot will also give a clear idea as to how different factors affect the development of clubroot and how these factors interact. This will allow future researchers to develop new and more effective treatments against clubroot. The development of a rapid quantitative PCR test will also be a great research tool and of great value to growers because it will allow rapid identification of fields infected with *P. brassicae* spores and this will help growers to decide which control treatments they should apply to the soil for the maximum level of disease control for the minimal cost.

CHAPTER 2 – PATHOTYPES OF *PLASMODIOPHORA BRASSICAE* IN SOILS FROM ABERDEEN, FIFE AND LINCOLNSHIRE DETERMINED USING THE EUROPEAN CLUBROOT DIFFERENTIAL (ECD) SERIES

Introduction

Selective speciation of *P. brassicae* spores was suspected by Appel and Werth as early as 1910, but Honig was the first person to demonstrate individual pathogenic races of *P. brassicae* in 1931 (Williams 1996). He discovered that spores differentially infected brassica hosts based on their pathogenicity. Attempts to classify *P. brassicae* into named physiological races began in the 1950's as clubroot researchers realised that problems due to pathogen variation and host resistance made it difficult to compare results gained in studies on the life cycle and in the development of control measures against the organism.

Many different types of test were independently developed to discover which pathogenic races of spores were being used in experiments. Ayers (1957) developed a test which included some well known clubroot resistant host plants as well as a number of wild crucifers. Seaman *et al.,* (1963) expanded Ayers' method to include a resistant cabbage 'Badger Shipper.' The use of wild crucifers in a test of physiological specialisation of the spores was discouraged because of the difficulty in obtaining genetically uniform seed from wild hosts (Williams, 1966). Williams (1966) suggested that breeders of economic crucifers needed to determine which races of the organism was present in screening programs and developed a test based on the reaction to infection of four commercial crucifer cultivars. This test grouped *P. brassicae* into 16 races based on their ability to infect test hosts.

The European Clubroot Differential (ECD) series was developed in 1974 (Buczacki *et al*., 1975) and was introduced in an attempt to provide a single, internationally acceptable set of differential hosts so that scientific work on clubroot could be coordinated (Jones and Ingram, 1982). The seed for this test is obtained from Horticulture Research International, Wellesbourne and therefore all researchers use seeds from the same genetic source of plants. The ECD series includes most of the sources of clubroot resistance that were available and was being exploited in plant breeding during the 1970's (Crute *et al.,* 1983). It is made up of five hosts each of

Brassica rapa, *B. napus* and *B. oleracea* (Table 2.2). Hosts 01 – 04 (Dutch stubble turnips) and 06 – 10 were included in the test because they were specifically bred for the purpose (Buczaki *et al.,* 1975). The major mechanism of resistance of these cultivars/cultivars towards *P. brassicae* is differential and is probably oligogenic (Crute *et al.* 1983). Host 05 was described as a universally susceptible host and is used as a control for the ECD test as a whole to check for the viability of spores used in the test (Buczaki *et al.,* 1975). Differential hosts 11 – 15 were more arbitrarily selected. The *B. oleracea* genotypes are generally non-differential in their resistance to *P. brassicae* and any resistance that they possess may be due to multiple genes (Dixon and Robinson 1986). Host 11 may be the only one of the *B. oleracea* hosts the test to be truly differential (Crute *et al.* 1983). Host 13 (a parent of host 11) was taken from Williams' 1966 test; host 12 is a well known clubroot resistant parent and host 14 is the most susceptible of the *B. oleracea* cv. *capitata* hosts. Host 15 is a Dutch 'clubroot resistant' curly kale cultivar (Buczaki *et al.,* 1975). A numerical code, consisting of three numbers representing the observed susceptibility of hosts within each of these three species is then assigned to each different pathogen population tested by adding denary values (assigned to each plant) of hosts within a species group showing a susceptible reaction (Donald *et al*., 2006). The ECD series is now commonly used as the main method of classifying spore populations of *P. brassicae* throughout the world.

In this study, the ECD series was used to determine the differential pathogenicity of *P. brassicae* spores from field soil in Fife, Aberdeen and Lincolnshire. The tests were carried out to discover the different races of spores that had been present in clubroot infested field soil that was to be used in trials to examine the effectiveness of clubroot control measures (Chapter 4). A spore population that had been used in glasshouse trials (Chapter 3) and which had originated from a mixture of clubroot-infested field soil from around Fife also was also tested for pathogenicity. The spore populations in the soils tested had not been determined in the past, therefore, the ECD test was used to determine the range of pathogenicity genes within these spore populations. This was to determine whether there was a difference in populations between soils which could

possibly explain the reason for any differences in the level of control achieved using the same treatments in different trial sites.

Materials and methods

Source of soil used for bulking-up of pathogen inocula

Five soils were tested from a range of geographical locations, selected because they were known to contain high levels of clubroot (Richard Haacker, Peter Gladders, pers. comm.) and because the soils would be used for testing clubroot control measures in both the glasshouse and field (chapters 3 and 4). Each soil was tested for pH before ECD testing commenced. Clubroot infested soil was collected from 'P & Q' beds at the Scottish Agricultural College, Craibstone Estate, Aberdeen. These beds which have been established for decades are used to test clubroot resistant rape cultivars at the College. The soil type was granitic till and had a pH of 5.2. Clubroot infested soil was also collected from Kirkmay farm, Crail, Fife, Scotland. This was a sandy loam soil with a pH of 7.5 and had been intensively cropped with vegetable brassicas (mainly from the species *B. oleracea*) over a number of years. Soil was also collected from Barnsmuir farm, Crail, Fife, Scotland. This was also a sandy loam soil with a pH of 6.5 and had also been intensively cropped with vegetable brassicas over a number of years. Finally, clubroot infested soil from Lincolnshire was collected from a site called Kirton. The soil type was fine silty loam, soil series: tanvats. The pH was 7.6 and had the following cropping history: 2006, calabrese; 2005, cauliflower; 2004, potatoes; 2003 calabrese. The grid references of these field sites can be seen in table 2.1. A mixture of clubroot infested soil from various fields used to grow vegetable brassicas around Fife was also tested. The soil was mixed because the soil was from fields that were tested for the presence of clubroot using a bait plant test (see below) by the crop clinic at SAC Edinburgh. If the soils tested were shown to cause high levels of disease on the roots, the soil was saved and mixed together to form a soil sample that was used as a 'positive control' soil in future bait plant tests. The soil type and cropping history was therefore mixed and unknown. The initial pH of the mixture of soil was pH 6.4.

Table 2.1. Grid references of field sites from which soil samples were taken

Farm name	Location	Grid reference
Craibstone Estate	Aberdeen	NJ 877 104
Barnsmuir	Fife	NO 596 062
Kirkmay	Fife	NO 604 073
Kirton	Lincolnshire	TF 296 360

Sampling of soils from field

The soil samples used in the ECD tests was sampled randomly, taking soil from numerous sampling points throughout the trial area (see Chapter 4) to a depth of approximately 10 cm. Approximately 5 kg of soil was sampled in this way. Sub-samples of each 5 kg soil sample were used for the replications in the ECD tests.

Bulking-up of pathogen inoculum

The soil from the field sites was placed in plastic seed trays with drainage holes (20 x 14.5 x 5.5 cm). Each of these trays was placed singly into a larger tray with no drainage holes (33.5 x 21 x 5.5 cm). Cross contamination was prevented by leaving space between each different tray. Twenty seeds of untreated Chinese cabbage cv. SB1 Kilo (from Steve Hove Seeds Ltd) were placed in the soil along 4 trenches, 5 seeds per trench. The trays were put on raised benches in the glasshouse and were watered daily by pouring water into the larger trays without holes. The glasshouse air temperature was set at 18^{o}C. After 6 weeks, the resultant galls were washed free of soil and were put in plastic bags and stored at -20^{o}C until required.

Preparation of inoculum

To prepare spore suspensions for use in the ECD test, the bulked-up galls of Chinese cabbage cv. SB1 Kilo were thawed under cold running water and were then homogenised in distilled water using a pestle and mortar. The resultant liquid was filtered through eight layers of muslin by diluting with more distilled water. The filtrate was centrifuged at 100 x g for 5 minutes to pellet plant debris and large starch grains.

The supernatant was then centrifuged at 6000 x g for 15 minutes to pellet the spores. The spore pellet was then re-suspended in 1 ml distilled water and the spore concentration was determined using a haemocytometer.

Inoculation of soil to be used in the ECD test

Plastic seed trays with drainage holes (20 x 14.5 x 5.5 cm) were filled with John Innes No. 2 potting compost, pH 5.5. Each of these trays was put into a larger tray with no drainage holes (33.5 x 21 x 5.5 cm). To inoculate the compost, a slurry was made by putting 150 ml of JI No. 2 compost into a plastic beaker and then adding a 20 ml spore suspension containing 10^8 spores/ml to it. Tap water was added to make the slurry up to a volume of 200 ml. This gave a final spore concentration of 10^7 spores/ml in 200 ml of slurry. This level of inoculum was chosen because it has been shown to give 100% infection in previous glasshouse trials (Harling and Kennedy, 1991). This slurry was mixed thoroughly and was poured along 4 shallow trenches in the soil, 50 ml of slurry along the length of each trench. The surface of the soil was flattened using a spatula.

ECD host plants

Seed of the 15 ECD hosts was obtained from Horticulture Research International, Wellesbourne. Ungerminated seed of these hosts were sown on the slurry, 20 seeds of each host per tray (5 seeds along the length of 4 trenches in the soil). The seeds were lightly covered with some fresh JI No. 2 compost. Two trays were used per ECD host so that 40 seeds of each ECD host were exposed to each source of inoculum. Growing conditions were indicated as above. The ECD tests were carried out between the months of June and August.

Clubroot assessment

Six weeks after the ECD host seeds were planted into the inoculated soil, plants were removed from the trays and the soil was washed from roots using running tap water. Clubroot severity was assessed using a 0 – 3 scale: 0, no swelling visible; 1, very slight swelling usually confined to lateral roots; 2, moderate swelling on lateral and/or tap

roots; 3, severe swelling on lateral and/or tap roots. A disease index (DI) was calculated for each host based on this scaling and used to assign a host reaction type (resistant DI < 33, susceptible DI > 33). The disease index of 33 was chosen as a resistant reaction because a resistant reaction is considered as a reaction where *P. brassicae* does not enter the root cortex and therefore does not cause clubbing on the tap root (Voorrips and Visser 1993). If all of the plants tested had a severity rating of 1 (considered as a resistant reaction), a disease index of 33 would be given based on the calculation below.

The DI was calculated using the following equation:

$$DI = \frac{[n0 \times 0] + [n1 \times 1] + [n2 \times 2] + [n3 \times 3]}{n0 + n1 + n2 + n3} \times \frac{100}{3}$$

where $n0$ is the number of plants with a clubroot severity rating 0, $n1$ is the number of plants with a severity rating 1, etc after the method of Donald *et al.,* (2006).

Determination of pathogen race

A numerical code, consisting of three numbers representing the observed susceptibility of hosts within each of the three species of the ECD series was assigned to each different pathogen population tested by adding the denary values of hosts within a species group showing a susceptible reaction (Table 2.2). For example, an ECD code of 1/15/3 would represent a pathogen population that caused a susceptible reaction on *B. rapa* differential host 01, *B. napus* differential hosts 06, 07, 08 and 09, and *B. oleracea* hosts 11 and 12.

Table 2.2. European Clubroot Differential series with associated host numbers, binary and denary values (after Buczacki *et al.* 1975, common names from seed packets).

Differential Number	Host Name	Binary Value	Denary Value
	20 Chromosome group (*Brassica rapa*)		
01	cv. *rapifera* line aaBBCC	2^0	1
02	cv. *rapifera* line AAbbCC	2^1	2
03	cv. *rapifera* line AABBcc	2^2	4
04	cv. *rapifera* line AABBCC	2^3	8
05	cv. *pekinensis* cv. Pe-Tsai	2^4	16
	38 Chromosome group (*B. napus*)		
06	Fodder rape cv. nevin, line Dc101	2^0	1
07	Giant rape – commercial, line Dc119	2^1	2
08	Giant rape – selection, line Dc128	2^2	4
09	New Zealand resistant rape, line Dc129	2^3	8
10	Swede cv. Wilhelmsburger, line Dc130	2^4	16
	18 Chromosome group (*B. oleracea*)		
11	cv. *capitata* cv. Badger Shipper	2^0	1
12	cv. *capitata* cv. Bindsachsener	2^1	2
13	cv. *capitata* cv. Jersey Queen	2^2	4
14	cv. *capitata* cv. Septa	2^3	8
15	cv. *fimbriata* cv. Verheul	2^4	16

Results

The ECD series was used to determine the differential pathogenicity of *P. brassicae* in fields in Aberdeen, Fife, Lincolnshire and in a mixed source of soil collected from fields around Fife. The disease index of each host reaction to each of the spore inoculum and the triplet code assigned to each soil site can be seen in Table 2.3. All inocula were exposed to 40 plants of each host which is more than the minimum of 30 plants as suggested by Buczacki *et al*., (1975).

Table 2.3. Reactions of the ECD hosts to some UK collections of *Plasmodiophora brassicae.*

ECD host	Disease Index for each host reaction				
	Aberdeen, 'P & Q' beds	Fife, Barnsmuir	Fife, Kirkmay	Fife, mixed soils	Lincolnshire, Kirton
01	88	0	0	3	0
02	17	0	3	0	0
03	0	0	4	0	0
04	0	0	0	0	0
05	100	90	100	100	3
06	100	6	100	62	4
07	100	100	62	100	24
08	86	0	100	100	0
09	100	5	94	100	2
10	57	0	37	41	0
11	65	0	73	90	0
12	78	78	73	76	4
13	53	85	84	91	11
14	39	86	100	100	0
15	41	65	76	44	0
ECD code*	17/31/31	16/2/30	16/31/31	16/31/31	0/0/0

* Race number assigned on the basis of susceptible reactions (DI > 33).

The race of *P. brassicae* spores present in the Aberdeen soil was classed as 17/31/31, Barnsmuir farm in Fife was classed as 16/2/30, Kirkmay farm in Fife was 16/31/31 and Kirton farm in Lincolnshire was 0/0/0. The clubroot races from a mixed source of Fife soil was classed as 16/31/31. Differential host 05 (*B. rapa* cv. *pekinensis* cv. Pe-Tsai) which is not the usual universally susceptible host 05 (normally the cultivar Granaat), was susceptible to all the spore inoculum tested with the exception of the Lincolnshire inoculum. The differential host 05 was provided by Horticulture Research International, Wellesbourne and was given in place of the unavailable cultivar Granaat to provide the same result as the cultivar Graanat. The Lincolnshire inoculum gave no susceptible reactions on any of the host plants as no disease index on any of the hosts exposed to

this inoculum was above 33. Virulence towards the *B. oleracea* group (hosts 11-15) was consistently high among the Scottish inocula with the exception of host 11 which showed no disease when exposed to the Barnsmuir inoculum. The virulence towards the *Brassica rapa* group (hosts 01-05) was extremely limited in all of the inocula tested with the exception of the control host 05 and host 01 which was susceptible to the Aberdeen inoculum. None of the inocula tested was capable of causing a susceptible reaction on host 04. Hosts 09 - 15 produced high numbers of intermediate reactions in all of the Scottish pathogen populations tested and the disease indices of these hosts were very varied among all the different sources of inocula tested. There were no differences between the results of the two different trays of hosts used for each inoculum.

Discussion

This study has shown that there are different pathotypes of *P. brassicae* present in field soils around the UK. Differences between field populations of *P. brassicae* have been shown by many workers including Dobson (1983); Donald *et al*., (2006); Manzares-Dauleux *et al*., (2001); and Voorrips and Visser (1993). The two fields in Fife that had different pathotypes of *P. brassicae* in their soil are very close in distance. Barnsmuir Farm is on the sea front in Crail, and Kirkmay Farm is on the opposite side of a road just a few 100 m away from Barnsmuir Farm (see Figure 1 in Appendix 2). The results show that there can be large differences in *P. brassicae* pathogens in fields that have the same soil type and are located very close to each other. This may suggest that the differences in pathotypes between these soils is not due to climatic conditions which are similar in both fields, but are due to other factors such as crop rotations, cropping history and management of the two fields.

Crop rotation as a reason for differing pathotypes between fields in close proximity

Voorrips (1995) showed that the existence of genetic variation for pathogenicity of *P. brassicae* in field populations can be due to the response of selection pressure. He showed that passing a spore population through a partially resistant crucifer cultivar five times gave an isolate with an increased pathogenicity towards this host. In the absence

of the resistant host, pathogenicity was reduced. Jones *et al.,* (1982) also showed that the selection of pathotypes in a population can occur when one inoculum is passed through a range of differential hosts. Dixon and Robinson (1986) found that spores from different plants grown in the same field but propagated in different hosts showed different results when spores extracted from their galls were tested on the ECD host plants. Crute *et al.,* (1983) also put a field spore population through the same host plant for 7 generations and showed that the clubs got heavier with each generation. This demonstrated that there was an adaptation or increase in pathotypes that were more able to infect this host. The type of brassicas grown in the two Fife fields may therefore have been different to each other and the *P. brassicae* populations present in each field could have become adapted to the host plant species that were cropped regularly in them. This would cause the spore populations in the field to differ from each other, resulting in the differences shown in the ECD test.

Soil pH as a reason for differing pathotypes between fields in close proximity

Another explanation for the difference in spore population between the two Fife field soils could be as a result of the initial pH of the soil from which the inocula was sourced. Kirkmay soil had a higher initial pH than the Barnsmuir soil and it has been suggested that different races of *P. brassicae* spores may have varying tolerance to pH (Colhoun, 1953). The high pH conditions may therefore have caused the high pH tolerant races present in the soil to become dominant and preferentially infect the Chinese cabbage host plant used for the initial bulking-up of inoculum to be used in the ECD test, resulting in a different ECD outcome between the fields.

Differences in soil sampling as a reason for differing pathotypes between fields in close proximity

It was first shown by Tinggal and Webster (1981) that field isolates were not genetically uniform and that field populations of *P. brassicae* have clear differences in pathogenicity within field sites. They also showed that pathotypes can even vary within the same clubs. Jones *et al.,* (1982) and Manzares-Dauleux *et al*., (2001) both showed

that different single spore isolates from one population of *P. brassicae* could give different reactions with the ECD test. Therefore, soil samples obtained from different parts of the same field may contain spore populations with unique and different pathogenicities. This could suggest that another reason why the spore populations from the two Fife fields show different pathogenicities.

These facts suggests that the characterisation of a field isolate using a differential test series is in fact only valid for the inoculum used in the test. This means that the ECD test cannot be used to definitely assign a race number to the spore population in a field but could be used to give a general idea of the main pathogenic types present in the soil.

Fife spore race 16/31/31

The 16/31/31 race seen in both the Kirkmay Fife soil and from the mixed source of soil from fields around Fife is the same spore population described by Harling and Kennedy (1991) as coming from a field near Aberdeen in 1991. Similar results were shown by Dixon and Robinson (1986) in ECD experiments on soil from the same land from which the 'P & Q' bed soil was taken in this study (Scottish Agricultural College, Craibstone, Aberdeen). The Dixon and Robinson ECD experiments were carried out between 1978 and 1982. *Plasmodiophora brassicae* races from this soil were all classed as either 16 or 30 for the *B. rapa* group, 19 or 31 for the *B. napus* group, and13, 14, 30 or 31 for the *B. oleracea* group. One of the spore populations was classed as 16/31/31. The fact that this same spore population has been found throughout Scotland could suggest that this spore population contains the general range of pathogenicity of isolates in Scotland. The mixed spore population from the mixed soil source would be expected to contain a wide range of spore races and therefore may demonstrate the widest range of pathogenic races in the soil. The knowledge that the general range of pathogenicity of the isolates in Scotland using the ECD test is 16/31/31 could be useful to plant breeders who could use this isolate in a screening program that tests for disease resistance. It also suggests that there has been no major change in the pathogenicity of Scottish races of *P. brassicae* towards the ECD series hosts over the space of 28 years.

Aberdeen spore race 17/31/31

The soil used as a source for the Aberdeen inoculum was taken from a field that is used to test clubroot resistant rape lines. All of the rape hosts (*B. napus*) exposed to the Aberdeen soil inoculum gave a disease index of 100, with the exception of host 08 which gave a disease index of 86. The Aberdeen soil had a slightly higher disease index for the *B. napus* hosts than the Fife populations taken from soil where *B. napus* is not normally grown. This is more supporting evidence for the fact that spore populations exposed to certain plant hosts become more pathogenic towards these hosts. The land where mainly *B. oleracea* plants had been planted, (i.e. the Fife soils) had pathogen populations that caused a slightly higher level of disease in the *B. oleracea* hosts compared to the Aberdeen inoculum, indicating that the population of *P. brassicae* present in the Aberdeen soil appeared to be adapted towards causing disease on the *B. napus* hosts and the Fife populations were adapted to infecting their usual hosts of *B. oleracea*. The patch of land where the Aberdeen soil was taken from is known for a containing a very aggressive population of clubroot spores which is why it is used in the testing of clubroot resistant rape lines (Elaine Booth, pers. comm.). A susceptible reaction on host 01 is the main difference between the race of this spore population to other Scottish populations tested (Dixon and Robinson, 1986; Harling and Kennedy, 1991). By carrying out the ECD test on this spore population, the pathotype of this aggressive race has been classified but further experiments may be needed to clarify whether it is more aggressive than other spore races in Scotland.

Lincolnshire Kirton spore race

The ECD test on the Lincolnshire soil showed some low levels of clubroot infection on five of the ECD hosts. There was a small amount of infectivity within these spores but not enough to class any of the host reactions as susceptible. Crute *et al.,* (1983) said that at least one of the ECD hosts should have a DI of greater than 80 to show that the spore inoculum is viable enough for carrying out the test. If it is lower, the test should be carried out again with more inoculum added. The ECD test on this spore population was carried out at the same time as the Fife soil ECD tests using the same level of inoculum,

showing that conditions in the glasshouse were conducive for clubroot gall formation. The spore population from the Lincolnshire soil used in this test could therefore be classed as non-viable. This is despite the fact that calabrese plants planted in the field where this soil was sampled from were badly affected by clubroot symptoms. There are no convenient methods for checking for spore viability and the viability of the spores was not checked before carrying out any of the ECD tests. The ECD test was not carried out on this soil using a higher level of inoculum because it was difficult to bulk-up enough inoculum from this spore population to be used in the test and there were also time constraints. The Lincolnshire soil had a very high pH when it was first used to bulk up the Chinese cabbage galls as a source of inoculum. The pH of the soil was pH 7.6 and pH 7.2 is generally classed as the pH at which *P. brassicae* spores cannot progress beyond the root hair stage of its life cycle and therefore cannot go on to form galls on the plant roots (Webster and Dixon, 1991). This high pH did not prevent galls from being formed as enough inoculum was bulked-up in the Chinese cabbage plants from the initial soil. However, the high pH that they were exposed to in this soil may have influenced the spores in some way so as to make them less infective on future host plants. If the DI susceptible limit was reduced to 20 or below, the Lincolnshire spore population would be classed as 0/2/0. There is no agreed standard DI cut-off point for determining that a host reaction is resistant or susceptible but it is generally considered that consistent scores of 0 and 1 on the gall grades is classed as a resistant reaction (Voorrips and Visser, 1993). Therefore, consistent grade 1's on all of the plants tested would give a DI of 33 using the DI calculation (see explanation in materials and methods). This was the reason that in this study, the cut-off point for a resistant reaction was classed as 33. A cut-off point for a resistant reaction was also classed as 33 by Donald *et al.,* (2006). However, Bowers (1982) classed a DI of more than 10 as susceptible which would then give the Lincolnshire spore race a classification of 0/2/4. Crete and Morgan (1980); Harling and Kennedy (1991); and Some *et al.,* (1996) used a cut-off point for resistance of 25. (Voorrips and Visser 1993) used a cut-off point of 50. Crute *et al.,* (1983) used DI frequency distributions to determine the cut-off point of a resistant reaction. The DI cut-off point that related to a resistant reaction would be

dependant on the DI calculation used, but based on the research by Voorrips and Visser (1993) which indicated that a resistant reaction could be classed as root hair infection only, a disease index cut-off point which related to a level of disease that showed no clubbing in the taproot of any host plant would be the most useful measure of resistance.

Resistance towards *B. rapa*

The field isolates tested were virtually all non-pathogenic towards the *B. rapa* differential hosts except from the control host 05. The same results have been shown with Australian isolates (Donald *et al.,* 2006). Toxopeus *et al.,* (1986) also reported that a susceptible reaction on only host 05 of the *B. rapa* hosts is the most frequent reaction of European isolates. The *B. rapa* hosts are Asian vegetables. These vegetables may never have been grown commercially in Scotland and therefore strong virulence towards this group may not have developed.

Intermediate disease reactions of some ECD hosts

The *B. oleracea* hosts showed an intermediate disease reaction to the inoculum that was tested. These intermediate reactions on the *B. oleracea* hosts agree with the observations of other workers (Crute *et al*., 1983); (Toxopeus *et al.,* 1986); and (Donald *et al.,* 2006) and gives more evidence to indicate that the *B. oleracea* hosts do not have differential resistance but that the resistance may be due to the involvement of many genes. Another explanation for intermediate reactions on differential hosts is that if a mixed population of spores is being tested, a pathotype occurring at a low frequency within the population may infect only a few plants of a susceptible ECD host (Dobson *et al.,* 1983). Therefore, pathotypes present in low frequencies in field populations may not actually be detected by the ECD test.

Effect of mixtures of pathotypes in a population on the ECD test results

Jones *et al*., (1982) tested two different single spore isolates from the same spore suspension against the ECD hosts, and found that the two isolates gave different reactions from the spore suspensions from which they were derived. When the two single spore isolates were mixed together and then tested on the ECD hosts, spores of

one isolate restricted infection by the spores of the other isolate. This shows that the most pathogenic pathotypes in a spore population will be detected by the ECD test (depending if there is a sufficient level of that isolate present) but pathotypes present in low frequencies in field populations or pathotypes that are less pathogenic against the differential hosts may not be detected. This means that the ECD results may only indicate the differential pathogenicity of the major components of the population and more complex and/or minor components may not be detected, especially if they occur at a low frequency (Jones and Ingram, 1982). Therefore using spore populations of known pathogenicity (as established by the ECD test) in breeding programs will not guarantee effective selection of resistance against low frequency pathotypes. Spores in a mixed spore population may compete with each other by competing for sites of infection on a host root and therefore, if a certain pathotype is at a low frequency in a population it will have a lower probability of attaching to a root hair. Cross-pathotype interaction may also occur as a result of induced resistance, stimulated following unsuccessful infection by one pathotype (Some *et al*., 1996). Another experiment by Jones *et al*., (1982) showed that continuous cropping with Badger Shipper cabbage in a soil increased the pathogen virulence towards the host, but that the effectiveness of the spore population to infect the host was reduced after a three year break without this host in the soil. Crute *et al.,* (1983) suggested that this may indicate that more virulent strains within a population do not compete well with other pathotypes in the absence of selection. Jones *et al*., (1982) mentioned that the selection of certain pathotypes by host plants has implications for agricultural practices because the infection potential in a certain field depends on the cultivated cultivars and wild crucifers in the field. Therefore, the rotation of crops and cultivars should be considered as an opportunity to prevent the continuing selection of yet more virulent strains.

Problems with ECD test

The ECD test is time consuming, labour intensive and subject to environmental conditions as shown by Some *et al.,* (1996) who demonstrated differing results between winter and spring tests. The limitations of the set have been commented on many times

but attempts to refine or improve it have not been made (Some *et al.,* 1996). Contradictory results and conclusions about the ECD test may be explained in part by the lack of consistency with regard to the methods used by individual laboratories. For example, inoculation methods and inoculum concentrations vary across all the ECD literature. It has also been shown that some differential hosts require a higher concentration of spores than others to give the same incidence of clubroot (Jones *et al*., 1982). This means that the infection of all the plants of the universally susceptible host (05) cannot be taken as evidence for the reliability of that test for other hosts. There are no set standards on the amount of inoculum used in ECD tests although Buczaki *et al*., (1975) suggested that a minimum level of 10^8 spores/g soil should be used. To inoculate enough soil to grow 30 plants in with this high a level of spores requires a large amount of galled root material. For example, throughout the glasshouse work carried out in this study, 1 g of galled Chinese cabbage root was shown to contain approximately $10^7 – 10^8$ spores. Therefore, to inoculate only one tray of soil of containing 500 g of soil with 10^8 spores/g soil would require approximately 5 kg of galled root material. This may in part, account for the variability in the levels of inoculum used by researchers, with some using as little as 10^6 spores/g soil (Donald *et al*., 2006). A standard inoculum level should therefore be used universally to overcome the differences in results among tests with different spore loads.

Differing interpretations of the varying results obtained and subsequent conclusions may also have led to doubts about the value of the ECD test. Different disease index calculations and cut-off points for resistant reactions have been used in the ECD tests (Voorrips and Visser 1993; Some *et al.,* 1996; Donald *et al.,* 2006). The number of individual plants used has varied among workers (Donald *et al.,* 2006; and Jones *et al.,* 1982), and finally, the subjective scoring system of the roots is arbitrary and cannot be universally applied (Crute *et al.,* 1983).

The inoculum also has to be fully representative of a plot of land and so must be sourced using appropriate sampling procedures. Also, if clubs are being used as the inoculum

source, a large number of galls should be used to prepare the inoculum as any single gall may not contain the same spore population as other galls. An additional difficulty with the ECD test is the lack of genetic uniformity in the differential ECD hosts due to self-incompatibility and a certain extent of cross-pollination. Williamson worked on the same ECD *B. napus* plants with many pathogen populations and found evidence of non-differential interactions (Voorrips and Visser, 1993). The genetic homozygosity of some ECD entries is therefore doubtful and could lead to contradictory results in the test. Selection for specific pathotypes could also take place in bulking up the spore inoculum using the Chinese cabbage host. Bulking of isolates may therefore mask the variability present within the spore populations and may result in an artificially homogenous spore inoculum (Asano *et al.,* 2006).

Alternatives to the ECD test

Few molecular methods have been developed to determine the extent of genetic variation within pathogen populations (Asano *et al.,* 2006). The limited tests on spore genetic variation using molecular markers have shown that molecular markers can distinguish more spore races than can the ECD host plants (Asano *et al.,* 2006). Therefore, the number of races of *P. brassicae* are still unknown and natural *P. brassicae* variation in virulence is probably much greater than is already described. The development of microsatellite-based molecular markers for the identification of different *P. brassicae* races may therefore help towards finding a faster and more reliable way of identifying *P. brassicae* isolates than the ECD test (Donald *et al.,* 2006).

Improvements to the ECD test

The best way to determine a spore population pathotype using the ECD test would be to use many single spore isolates extracted from spore suspensions of the inoculum to be tested. This would be extremely time consuming as all of the single spore isolates would have to be tested on all of the 15 ECD hosts. Also, single spore isolates have a variable success rate when it comes to infecting host plants (Jones *et al*., 1982). Many replications of the ECD test should be carried out for each inoculum source and

environmental conditions should be standardised. A standard method of inoculation and level of spore inoculum should be used throughout the world. The same disease index calculation and cut-off points for susceptible reactions should also be used so that results could be compared from many workers.

Conclusions

Field spore populations are made up of many different spore pathotypes with variation existing between and within field sites. The different pathotypes can become adapted to the different host plants that are planted in the soil and can interact with each other to differentially infect hosts (Jones *et al*., 1982). Therefore, reliable results from the ECD test cannot be obtained until the composition of pathotypes within populations and the interactions between the pathotypes are understood. Other limitations to the ECD test are that the test only identifies the most dominant pathotypes in the soil, low frequencies of pathotypes may not be detected by this test, and the presence of non-viable spores within a sample may affect the outcome of the test. The sampling method used to provide the inoculum for testing is also important because different soil samples from the same site may contain different pathotypes. The ECD test is useful in providing the knowledge of the general range of the most pathogenic pathotypes in a field population based on the sample tested, and this knowledge could be used in resistance genetic tests and in choosing breeding strategies to develop durable clubroot resistance (Asano *et al.,* 2006). Understanding the differences in spore races between field sites may also be useful in helping to understand why clubroot control measures are effective in some fields and not in others. Dominant pathotypes in soil could be determined using the ECD test, and then re-tested after treatments have been added to the soil to determine whether the treatments have had an effect on certain pathotypes in a population. The results of the ECD test on Scottish soil showed that the field isolates tested were virtually all non-pathogenic towards the *B. rapa* differential hosts except the control host 05. This result is similar to other European and Australian isolates. They were also highly pathogenic towards the *B. oleracea* hosts and this knowledge could be useful to

plant breeders who could use these isolates to develop clubroot resistant *B. oleracea* cultivars for use in Scotland.

CHAPTER 3- CONVENTIONAL AND NOVEL TREATMENTS FOR CONTROL OF CLUBROOT OF BRASSICAS: GLASSHOUSE EXPERIMENTS

Introduction

Current control measures for clubroot (such as adding lime to the soil to raise soil pH, adding the fertiliser calcium cyanamide, adjusting soil nutrition, and carrying out crop rotations) although all partially effective, do not give adequate or consistent clubroot control. Brassica growers require new sustainable control measures that can give consistent control, reduce spore levels in the soil and increase plant yields. Glasshouse experiments were carried out to examine the effectiveness of a wide range of conventional and novel treatments and treatment combinations for controlling clubroot. The aim was that if these treatments were effective in the glasshouse, they could be developed into new control measures for the field. With current pressure to reduce pesticide inputs, the experiments focused on using natural products and products that were seen as waste products from other industries that would normally be thrown into landfill. These products would be sustainable, cost-effective and their use would benefit both the brassica growing industry and the industry from which they were produced as waste.

Six glasshouse trials were carried out to test the effectiveness of various treatments at controlling clubroot and to determine whether any of these treatments would be suitable to use in a field environment. The first glasshouse experiment carried out in 2004 aimed to examine a wide range of initial treatments to determine the effectiveness of the various products at controlling clubroot on two different cultivars of calabrese. Treatments tested included conventional lime treatments, biocontrol agents, surfactants, fertilisers and fungicides.

A follow-up experiment in 2005 was aimed to refine the rates and timing of applications of the most effective treatments from the first initial screen and to test some new treatments such as plant extracts and waste products from industry (shell fish processing and mushroom growing industries).

The third experiment carried out in 2006 aimed to investigate combinations of treatments for possible additive effects. A follow-up experiment aimed to investigate the effectiveness of treatments when applied to the outside of module soil prior to planting. A trial was also carried out to examine the effect of adding oilseed rape meal at selected times before transplanting to control clubroot.

A final experiment was carried out in 2007 to examine the effect of natural soil microflora on clubroot disease control in field soil by adding various control treatments to *P. brassicae* inoculated field soil which had previously been autoclaved.

The effect of the treatments on disease level, fresh weight and general health of the plants were assessed to give an indication as to whether these treatments would be effective in a field environment. The discussion also explains why the treatments may have been effective at controlling disease and why some treatments were more effective than others.

Materials and methods

Preparation of resting spore suspensions

Resting spore suspensions of *P. brassicae* were prepared from clubroot galls from Chinese cabbage cv. Kilo that had been bulked up using the slurry method (see Chapter 2) from a mixed source of field soil from around Fife (see Chapter 2). The spore population was determined as 16/31/31 using the European Clubroot Differential (ECD) method (see Chapter 2). The root galls were homogenised using a minicraft drill in tap water, filtered through 8 layers of muslin using 20 – 25 ml tap water and centrifuged at 100 x g. The pellet from this step was discarded and the supernatant was spun at 6000 x g for 15 minutes to pellet the clubroot spores. The spores were then re-suspended in de-ionised water and a haemocytometer was used to count the spores.

Inoculation of soil

Plastic pots of 12.5 cm diameter were filled with approximately 500 g of John Innes No. 1 compost. The pH of this compost was 5.5. A 50 ml aliquot of the clubroot resting spore suspension (16/31/31) containing 10^6 spores/ ml was poured over the top of the soil to give a final concentration of 10^5 spores/g soil. The spore concentration had been measured using a haemocytometer. This level of spores was used because it had been shown to cause high levels of disease in experiments carried out by Harling and Kennedy, (1991).

Experimental design

Trial design was as randomised blocks consisting of four blocks, each block containing one pot per treatment and two positive controls and two negative controls. Two controls were used because plants within the same pot could vary in their level of disease. Therefore, the aim of having two controls was to reduce the effect on the treatment results of the unpredictable nature of clubroot development within the positive control pots.

Treatments

A large selection of treatments were analysed for their effectiveness against clubroot in these experiments. The treatments were chosen based on previous evidence that they had a clubroot controlling effect, or were chosen as it was thought that their components would have an effect on clubroot following a review of the clubroot literature. See tables 3.1 – 3.5 for a description of each treatment tested. Treatments were either in the form of a powder (P), were granular (G) or were a liquid (L).

Table 3.1. Description of lime treatments

Treatment	Supplier	Description
Calcium carbonate (P)	Buxton lime industries	TruCarb 295 is a fine powdered form of calcium carbonate
Calcium oxide (P)	Buxton lime industries	Calbux 90M is a very fine powdered form of calcium oxide
Calcium oxide gravel (G)	East of Scotland Growers Ltd.	Pebble-sized and shaped pieces of calcium oxide
LimeX 70 (P)	British Sugar Ltd.	LimeX 70 is a fine powdered form of calcium carbonate which is a by-product of the sugar beet processing industry
QuickCal (G)	PP Products Ltd.	Flaked calcium oxide

Table 3.2. Description of treatments supplying soil nutrients

Treatment	Supplier	Description
Biohumate (L)	Biotechnica Services Ltd.	An alkaline liquid based on humic acids and plant glycosides used to enhance nutrient uptake by plants
BioMagic (P)	Travena	Organic fertiliser based on seaweed extracts. Contains cytokinins
Bod Ayre seaweed (G)	Bod Ayre Products Limited	Seaweed granules made from Ascophyllum nodosum (Bladder weed)
Borax (P)	Sigma	A salt of boric acid also known as sodium tetraborate $Na_2B_4O_7.10H_2O$
Calcium nitrate (P)	Sigma	A soluble salt fertiliser $Ca(NO_3)_2$
Calcium silicate (P)	Sigma	Obtained by reacting calcium oxide and silica Ca_2SiO_4
Copper carbonate (P)	Longcliffe calcium carbonates	Powdered copper carbonate $CuCO_3$
Gypsum (P)	Sigma	Powdered calcium sulphate dehydrate $CaSO_4.2H_2O$
Perlka (G)	PP Products	A granular form of the fertiliser calcium cyanamide
Potassium tetraborate (P)	Biotechnica Services Ltd.	Powder $K_2B_4O_7.4H_2O$

Activation of biological control agents

The Pseudomonas treatments were grown on Kings Buildings (KB) agar plates (20 g neutralised bacteriological peptone, 1.5 g di potassium phosphate, 1.5 g magnesium sulphate heptahydrate, 15 g agar, 15 ml glycerol made up to one litre using de-ionised water) at 29^{o}C for 36 hours. A single colony was then added to 250 ml of KB media (as per KB agar recipe but without the agar added) in a 500 ml flask. This flask was aerated for 24 h at 29^{o}C. At this point, the colony contained 3 x 10^{9} colony forming units (CFU)/ ml. The required amount of media to get the correct bacterial concentration for the experiment (see Tables 3.7 and 3.8) was centrifuged at 12,000 x g for 15 minutes and the pellet was resuspended in 100 ml of de-ionised water to give the required concentration. The standard activation procedure for the Bactolife product was as follows: 875 ml of water at 30^{o}C, 100 g brown sugar, 20 g seaweed extract (Biotechnica), 5 g Bactolife. This mixture was dissolved well and aerated at 30^{o}C for 24 hours. It was then made up to 5 l using distilled water and the correct concentration of product needed was poured over the soil.

Table 3.3. Description of fungicides

Treatment	Supplier	Description
Amistar (L) Methyl(*E*)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate	Syngenta	Fungicide containing the active ingredient azoxystrobin (23.1%)
Ranman (L) 4-chloro-2-cyano-*N*,*N*-dimethyl-5-*p*tolylimidazole-1-sulfonamide	Belchim	Fungicide containing the active ingredient cyazofamid (40 %)
Shirlan (L) 3-chloro-*N*-(3-chloro-5-trifluoromethyl-2-pyridyl)-a,a,a-trifluoro-2,6-dinitro-*p*-toluidine	Syngenta	Fungicide containing the active ingredient fluazinam (38.4 %)

Table 3.4. Description of biocontrol treatments

Treatment	Supplier	Description
Bactolife DP104 (L)	Biotechnica Services Ltd.	Commercially available powder containing a mix of 20 different microorganisms such as Bacillus and Pseudomonas species, yeasts and fungi
Bactolife S (L)	Biotechnica Services Ltd.	Commercially available powder containing a mix of bacterial species only
Chitin (P)	Dr Michael Healy, Queens University, Belfast	Lactic acid bacteria-processed prawn shells
Pseudomonas chlororaphis PCL 1391 (L)	Leiden University	Bacterial strain producing the anti-fungal compound phenazine-1-carboxamide
Pseudomonas fluorescens WCS 365 (L)	Leiden University	Plant growth promoting rhizobacteria
Shell sand (P)	J & I Anderson	Crushed scallop and whelk shells
Spent mushroom compost	Greenmyre Mushroom Farm Ltd.	This was compost that has previously been used for growing mushrooms

Table 3.5. Description of surfactant, plant extracts, chitosan and disinfectant treatments

Treatment	Supplier	Description
Agral (L)	Syngenta	A non-ionic spray adjuvant
Oilseed rape meal (P)	Scottish Agricultural College, Aberdeen	A fine powdered meal – a waste product of oilseed rape oil extraction
Peppermint oil (L)	Linzoil	Oil extracted from the plant *Mentha x piperita*
Quillaja QL35 (L)	Desert King International	Quillaja QL 35 was an extract of quillaja plant saponins
Rhubarb leaves		Rhubarb leaves ripped into 1 cm^2 pieces
Rhubarb water (L)		Rhubarb leaves boiled in water for 10 minutes. Water was cooled and added to the soil
SIPeco (L)	DB ECOsystems	Disinfection fluid based on hydrogen peroxide
Softguard (L)	Travena	Chitosan oligosaccharin
Yucca SC:YF:60 (L)	Biotechnica Services Ltd.	Yucca was an extract of yucca plant saponins

Addition of treatments to pots

In the case of powdered and granular treatments (treatments marked with (P) or (G) in tables 3.1 – 3.5), the 500 g of inoculated John Innes No. 1 compost was emptied onto a plastic tray, the powder was mixed thoroughly into the soil and the treated soil was put back into the pot. In the case of liquid treatments (treatments marked with (L) in tables 3.1 – 3.5), these were poured over the top of the soil.

Rate of treatments

For treatments tested in the 2004 – 2006 experiments and their rates and time of addition see tables 3.6 - 3.8 respectively. Treatment rates were decided on by following product guidelines, by reviewing literature for rates that had controlled similar diseases in the past, and in the case of limes by calculating how much would be needed to raise the pH of the soil above pH 7.2 based on their neutralising value and the initial pH of the John Innes No. 1 compost. Rates were changed in each experiment to determine if lower or higher rates of each treatment would have a greater effect on clubroot control based on the results gained in previous experiments.

Table 3.6. Treatments, rates and times of soil application in the initial screening of treatments for clubroot control in two cultivars of calabrese: 2004

No.	Treatment	Rate/12.5 cm diameter pot	Rate/ha
1	Negative control		
2	Negative control		
3	Positive control		
4	Positive control		
5	Agral	100 µl in 100 ml water	0.1%
6	Bactolife DP104	87.5 ml water, 10g brown sugar, 2g seaweed extract 0.5 g Bactolife diluted with deionised water to 100 ml	70 kg/ha
7	Bactolife S	As Bactolife DP104	70 kg/ha
8	Biohumate	5 ml in 100 ml deionised water	5%
9	Biohumate + $CaCO_3$	5ml in 100 ml water + 1.875 g	5% + 260 kg/ha
10	Biohumate + Potassium tetraborate	5ml in 100 ml water + 0.015 g	5% + 21 ppm
11	Biohumate + $CaCO_3$ + Potassium tetraborate	5ml in 100 ml water + 1.875 g + 0.015 g	5% + 260 kg/ha + 21 ppm
12	Calcium carbonate	1.875 g	260 kg/ha
13	Calcium oxide	1.61 g	230 kg/ha
14	Calcium silicate	1.95 g	300 kg/ha
15	Chitin	5 g	1% (w/w)
16	Perlka	0.39 g	60 kg/ha
17	Potassium tetraborate	0.015 g	21 ppm
18	Potassium tetraborate + $CaCO_3$	0.015 g + 1.875 g	21 ppm + 260 kg/ha
19	Pseudomonas chlororaphis	1 x 10^9 CFU/ml in 100ml deionised water	1 x 10^9 CFU/ml
20	Pseudomonas fluorescens	1 x 10^9 CFU/ml in 100ml deionised water	1 x 10^9 CFU/ml
21	Quillaja extract QL35	1 ml + 99 ml deionised water	10,000 ppm
22	Ranman	0.028 ml in 100 ml deionised water	4 l/ha
23	Shirlan	0.021 ml in 100 ml deionised water	3 l/ha
24	Yucca extract SC:YF:60	0.06 ml in 100 ml deionised water	9 l/ha

All treatments were added to the soil one day before transplanting

Table 3.7. Treatments, rates and times of soil application of treatments in the experiment to refine the rates and addition times of the most effective treatments from the initial screening experiment: 2005

No.	Treatment	Rate/12.5 cm diameter pot	Rate (ha)
1	Negative control		
2	Negative control		
3	Positive control		
4	Positive control		
5	Bactolife DP104 *	0.007g	1 kg
6	Bactolife S *	0.007 g	1 kg
7	BioMagic	1.4 g	200 kg
8	Borax	0.03 g	24 kg
9	Borax + $CaCO_3$	0.03 g + 10.5 g	24 kg + 1.5 t
10	Borax + $CaCO_3$ + Perlka	0.03 g + 10.5 g + 0.39 g	24 kg + 1.5 t + 600 kg
11	Calcium carbonate	10.5 g	1.5 t
12	$CaCO_3$ + Perlka	10.5 g + 0.39 g	1.5 t + 600 kg
13	Calcium oxide	5.25 g	750 kg
14	CaO + Perlka	5.25 g + 0.39 g	750 kg + 600 kg
15	LimeX 70	70 g	10 t
16	Peppermint oil	0.07 ml in 100 ml water	10 l
17	Perlka	5.25 g	600 kg
18	*P. chlororaphis* *	1 x 10^4 CFU/ml in 100ml deionised water	1 x 10^4 CFU/ml
19	*P. chlororaphis* *	1 x 10^6 CFU/ml in 100ml deionised water	1 x 10^6 CFU/ml
20	*P. chlororaphis* *	1 x 10^8 CFU/ml in 100ml deionised water	1 x 10^8 CFU/ml
21	*P. fluorescens* *	1 x 10^4 CFU/ml in 100ml deionised water	1 x 10^4 CFU/ml
22	*P. fluorescens* *	1 x 10^6 CFU/ml in 100ml deionised water	1 x 10^6 CFU/ml
23	*P. fluorescens* *	1 x 10^8 CFU/ml in 100ml deionised water	1 x 10^8 CFU/ml
24	Quillaja	0.28 ml in 100 ml water	40 l
25	Ranman	0.028 ml in 100 ml water	4 l
26	Rhubarb (leaves)	35 g	5 t
27	Rhubarb (water)	7 ml in 100 ml water	1000 l
28	Shell sand *	35 g	5 t
29	Shirlan	0.021 ml in 100 ml water	3 l
30	Softguard	0.1 ml in 100 ml water	15 l
31	Spent Mushroom Compost 10 %*	70 g	10 t
32	Spent Mushroom Compost 30 %*	210 g	30 t
33	Yucca	0.01 ml in 100 ml water	1.5 l

* Treatments added to the soil two weeks before transplanting
All other treatments added to the soil one day before transplanting

Table 3.8. Treatments, rates and times of soil application of treatments in an experiment to examine the effectiveness of combinations of treatments for clubroot control: 2006

No.	Treatment	Rate/12.5 cm diameter pot	Rate (ha)
1	Negative control		
2	Negative control		
3	Positive control		
4	Positive control		
5	Amistar	0.021 ml	3 l
6	Amistar + *P.chlororaphis*[2]	0.021 ml + 10^6CFU/ml	3 l + 10^6CFU/ml
7	Bactolife DP104[2]	0.007 g	1 kg
8	Bod Ayre seaweed[2]	1.75 g	250 kg
9	Borax[3]	0.084 g	12 kg
10	Borax[3] + $CaCO_3$[1]	0.084 g + 10.5 g	12 kg + 1.5 t
11	Borax[3] + *P.chlororaphis*[2]	0.084 g + 10^6CFU/ml	12 kg + 10^6CFU/ml
12	Borax[3] + $CaCO_3$[1] + *P.chlororaphis*[2]	0.084 g + 10.5 g + 10^6CFU/ml	12 kg + 1.5 t + 10^6CFU/ml
13	Calcium carbonate[1]	10.5 g	1.5 t
14	$CaCO_3$[1] + Amistar	10.5 g + 0.021 ml	1.5 t + 3 l
15	$CaCO_3$[1] + *P.chlororaphis*[2]	10.5 g + 10^6CFU/ml	1.5 t + 10^6CFU/ml
16	$CaCO_3$[1] + *P.chlororaphis*[2] + Amistar	10.5 g + 10^6CFU/ml + 0.021 ml	1.5 t + 10^6CFU/ml + 3 l
17	$CaCO_3$[1]+ CaO[1]	10.5 g + 5.25 g	1.5 t + 750 kg
18	Calcium nitrate[3]	1.75 g	250 kg
19	Calcium oxide[1]	5.25 g	750 kg
20	CaO[1] + Shirlan	5.25 g + 0.021 ml	750 kg + 3 l
21	CaO[1] + *P.chlororaphis*[2]	5.25 g + 10^6CFU/ml	750 kg + 10^6CFU/ml
22	CaO[1] + Shirlan + *P.chlororaphis*[2]	5.25 g + 0.021 ml + 10^6CFU/ml	750 kg + 3 l + 10^6CFU/ml
23	Copper carbonate[3]	14 g	2 t
24	Gypsum[3]	14 g	2 t
25	LimeX[1]	28 g	4 t
26	LimeX[1] + Shirlan	28 g + 0.021 ml	4 t + 3 l
27	LimeX[1] + *P.chlororaphis*[2]	28 g + 10^6CFU/ml	4 t + 10^6CFU/ml
28	LimeX[1] + Shirlan + *P.chlororaphis*[2]	28 g + 0.021 ml + 10^6CFU/ml	4 t + 3 l + 10^6CFU/ml

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29	Perlka[3]	1.75 g	200 kg
30	Perlka[3] + QuickCal[1]	1.75 g + 5.25 g	200 kg + 750 kg
31	Peppermint[3]	0.07 ml in 100 ml water	1 l
32	Peppermint[3] + $CaCO_3$[1]	0.07 ml + 10.5 g	1 l + 1.5 t
33	*P.chlororaphis*[2]	10^6CFU/ml	10^6CFU/ml
34	Quickcal[1]	5.25 g	750 kg
35	Quillaja	0.28 ml	40 l
36	Quillaja + $CaCO_3$[1]	0.28 ml + 10.5 g	40 l + 1.5 t
37	Quillaja + *P.chlororaphis*[2]	0.28 ml + 10^6CFU/ml	40 l + 10^6CFU/ml
38	Quillaja + $CaCO_3$[1] + *P.chlororaphis*[+]	0.28 ml + 10.5 g + 10^6CFU/ml	40 l + 1.5 t + 10^6CFU/ml
39	Shell sand[1]	35 g	5 t
40	Shell sand[1] + *P.chlororaphis*[2]	35 g + 10^6CFU/ml	5 t + 10^6CFU/ml
41	Shell sand[1] + Shirlan	35 g + 0.021 ml	5 t + 3 l
42	Shell sand[1] + Shirlan + *P.chlororaphis*[2]	35 g + 0.021 ml + 10^6CFU/ml	5 t + 3 l + 10^6CFU/ml
43	Shirlan	0.021 ml	3 l
44	Shirlan + $CaCO_3$[1]	0.021 ml + 10.5 g	3 l + 1.5 t
45	Shirlan + *P.chlororaphis*[2]	0.021 ml + 10^6CFU/ml	3 l + 10^6CFU/ml
46	Shirlan + $CaCO_3$[1] + *P.chlororaphis*[2]	0.021 ml + 10.5 g + 10^6CFU/ml	3 l + 1.5 t + 10^6CFU/ml
47	SIPeco	10 ml in 100 ml water	10 %
48	Softguard[2]	1 ml in 100 ml water	15 l
49	Spent Mushroom Compost[1]	210 g	30 t
50	SMC[1] + *P.chlororaphis*[2]	210 g + 10^6CFU/ml	30 t + 10^6CFU/ml

[1] added to the soil two weeks in advance of transplanting
[2] added to the soil one week in advance of transplanting
[3] added to the soil five days in advance of transplanting
All other treatments were added one day in advance of transplanting

Plants

Calabrese plants grown in module trays (27 cm^3 per module) were used in all experiments. In the 2004 experiment, Calabrese cv. Marathon and Calabrese cv. Monaco modules (Syngenta seeds Ltd.) kindly provided by East of Scotland Growers were used in two separate experiments. In all other experiments Calabrese cv. Monaco (Syngenta seeds Ltd) was the only test plant. This plant cultivar was tested because of its commercial use in Scotland. Seeds of this plant were planted into module trays containing Levington M3 compost covered in vermiculite, one seed per 27 cm^3 module. The modules were grown for six weeks until the plants were approximately 10 cm in height, before being transplanted into the pots under standard growing conditions. In the 2004 experiment, four plants were transplanted per pot. In all other experiments, three plants were planted per pot. This was because using three plants per pot was observed to allow better root growth for analysis than four plants per pot. Pots were randomly arranged on a steel mesh bench covered in plastic sheeting in a glasshouse and each pot was placed in a 15 cm tray without drainage holes. The pots were watered every day by placing water into these trays and keeping the soil at saturation point. The temperature within the glasshouse was set at 18^{o}C. In the winter months, supplementary lighting was supplied by 400 W sodium lamps to create an 18 h day.

Analysis of plants

Six weeks after the plants had been transplanted into the pots, the plants were carefully removed from the pots and all soil was washed off the roots using running tap water. The roots were graded for clubroot severity on a scale of 0 – 3 (see Chapter 2). This 0 – 3 scale was then converted into a disease index using the calculation in Chapter 2. The water was squeezed from the roots and the root system was snapped off from the first node of the plant. The root and shoot system were both weighed for fresh weight. Then, all roots that were diseased were removed from the root system and weighed to give a measurement of the percentage of roots that were diseased. Shoot and root general vigour and appearance was also noted and scored.

Soil pH and extractable calcium measurements

In the 2006 experiment examining the effects on clubroot of combinations of treatments, the soil from each pot was collected at the end of the experiment after the plants had been removed from the pots. Soil from each replicate of a treatment was mixed together to create one sample and a random sample (500 g) of soil from this mixture was taken for pH and extractable calcium analysis. The pH was determined using distilled water and a pH electrode. The extractable calcium analysis was carried out by the Scottish Agricultural College Analytical Services Department. To determine the extractable calcium content of the soil, the soil was air dried and milled to pass a 2mm sieve. The extraction was done by the modified Morgans method developed by SAC specifically for Scottish acidic soils (Allen, 1974).

Adding treatments to the outside of modules

Six week old calabrese cv. Monaco modules were removed from their trays and the outside of the module soil was rolled in the powdered treatments so that all of the outside of each module was completely covered by the treatments. The treated modules were then transplanted into the inoculated soil in the 12.5 cm diameter pots in the usual way.

Oilseed rape meal experiment

The standard pots inoculated with 10^5 spores/g soil had 5 g of oilseed rape meal powder added to them at either 8 weeks, 4 weeks, 2 weeks before transplanting or at transplanting of the six week old calabrese cv. Monaco modules. All pots were inoculated with spores at the same time and were put on 15 cm trays without drainage holes and were watered every day. This meant that all pots had been exposed to the same water levels and temperature before the addition of the plants.

Experiment examining the effect of natural soil microflora on clubroot disease control in field soil: 2007

The 2007 field soil experiment was set out in a completely different way to the rest of the experiments. Field soil had been collected from a naturally clubroot infested field in Barnsmuir, Crail, Fife (see Chapter 4). Soil weighing 10 kg was put into autoclavable bags and was autoclaved at 121°C for 30 minutes. The soil was put into twenty plastic seed trays with drainage holes (20 x 14.5 x 5.5 cm). Two trays of soil had no spores added to them and were used as negative controls. Six trays were used as positive controls. Two of these trays had either 10^4 10^6 or 10^8 spores/g soil added to them in the usual way. Six trays had 10^4 spores/g soil added and the last six trays had 10^6 spores/g soil added. Treatments were then added to the soil (the equivalent rate of 1.5 t/ha of powdered calcium carbonate or 10^6 CFU/ml of *Pseudomonas chlororaphis* PCL 1391). After the treatments were added, 20 seeds of Chinese cabbage var SB1Kilo were put into each tray. These trays were put into larger trays without drainage holes and were watered from underneath. The glasshouse temperature was set at 18°C. Another tray containing John Innes No. 2 compost pH 5.5 with 10^6 spores/g soil was set up as another positive control and non-autoclaved field soil was also used as a control. After 6 weeks, the plants were removed from the soil and were analysed in the usual way (see Chapter 2).

Data analysis

Data were analysed by one-way ANOVA (in randomized blocks) in the initial general screening of treatments experiment (2004), the experiment examining the effect of natural soil microflora on clubroot disease control in field soil (2007), the experiment that involved adding lime to the outside of the modules, and the oilseed rape meal experiment. In the 2005 and 2006 experiments, data was analysed by ordinal regression analysis with factors (three cut-off points, by splitting the % disease interval from 0 – 100 into 3 arbitrary parts to give roughly equal probabilities for each level). Many of the treatments in the 2005 and 2006 experiments gave total disease control or very high

levels of disease and therefore the data were skewed and were unable to be analysed using ANOVA. Therefore, splitting the disease results into three equal categories allowed the results to be compared and analysed using ordinal regression analysis. This analysis was suggested by Chris Theobald, BioSS, Scotland. Analyses were carried out in Genstat for Windows (7th Edition) (Rothamsted Research).

Results

Tables containing the full results of all the experiments (disease, fresh weight and soil pH) can be found in Appendix 1. This appendix also shows the full statistical results for the experiments. The most significant results from the glasshouse experiments are reported in this chapter.

Effectiveness of treatments at controlling clubroot in calabrese cv. Marathon and calabrese cv. Monaco (2004)

In the 2004 experiment, plants of the cultivar Marathon were badly affected by aphids and therefore the results may have been affected. Plants of the cultivar Monaco were not so affected by the aphids and were considered the more robust cultivar of plants tested hence the use of the cultivar Monaco in all subsequent experiments. This was not a deliberate approach to find a suitable cultivar for use in the experiments. In the 2004 experiments, no clubroot disease was seen in the negative controls which showed that no contamination of the soil by spores had occurred by splashing of water from pot to pot in the glasshouse.

The statistical analysis examined the effect of treatments on disease index and fresh weight compared with the positive control plants since treatments would have to give an increase in yield compared to the positive controls to be worthwhile. The two control treatments within each block showed no significant statistical differences between each other and have been combined to give one result for each control in the reporting of the results.

Biohumate, calcium carbonate and potassium tetraborate: initial experiment 2004

Potassium tetraborate and the combination of Biohumate and potassium tetraborate had no effect on disease in either of the cultivars of calabrese tested (Figure 3.1). Figure 3.1 also shows that the combination of Biohumate and calcium carbonate significantly reduced disease in both cultivars. The combination of calcium carbonate and potassium tetraborate also significantly reduced disease compared to the control plants in both cultivars. The combination of Biohumate, calcium carbonate and potassium tetraborate only significantly reduced disease in the cultivar Monaco. None of the treatments significantly increased plant fresh weight compared to the positive control plants in either cultivar, however the combination of Biohumate and calcium carbonate significantly reduced the fresh weight of the plants in cultivar Monaco. The fresh weight results from these treatments can be seen in Appendix 1, Table 2. It was observed that roots exposed to the Biohumate did not grow out past the module compost.

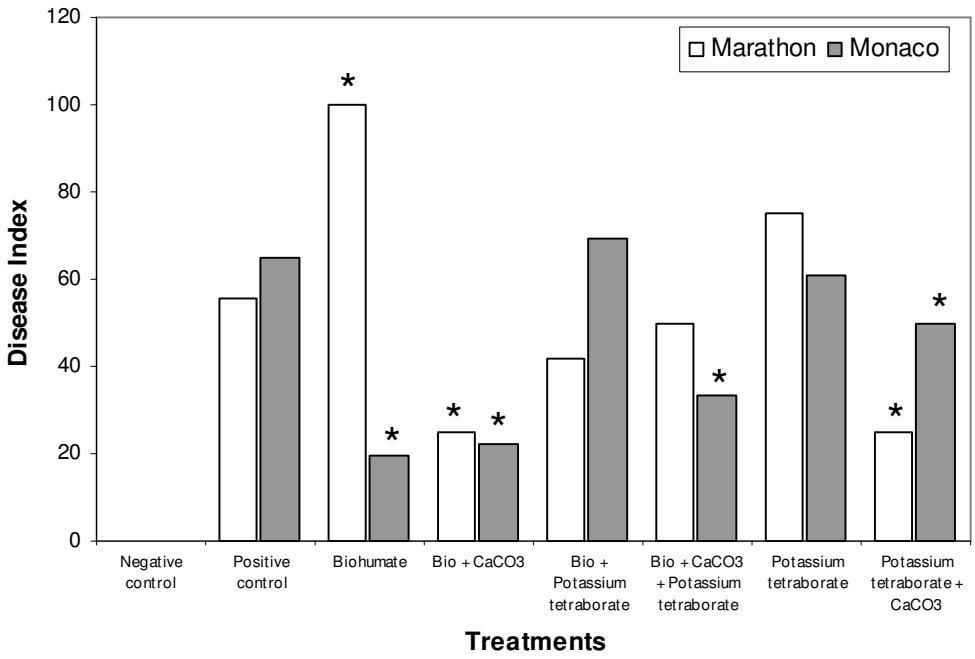

Figure 3.1 Effect of combinations of biohumate, calcium carbonate and potassium tetraborate on clubroot severity: cv. Marathon and Monaco (2004); LSD = *P<0.05

Surfactants: initial experiment 2004

The effectiveness of the two natural surfactants and the synthetic surfactant at controlling clubroot in the two cultivars of calabrese can be seen in Figure 3.2. All of the surfactants significantly reduced disease in the cultivar Monaco. The Yucca extract was the only treatment to significantly reduce the disease level in plants of the cultivar Marathon. None of the surfactants had a significant effect on plant fresh weight although the Quillaja extract had a more positive effect on plant fresh weight than the Yucca extract (see Appendix 1, Table 2).

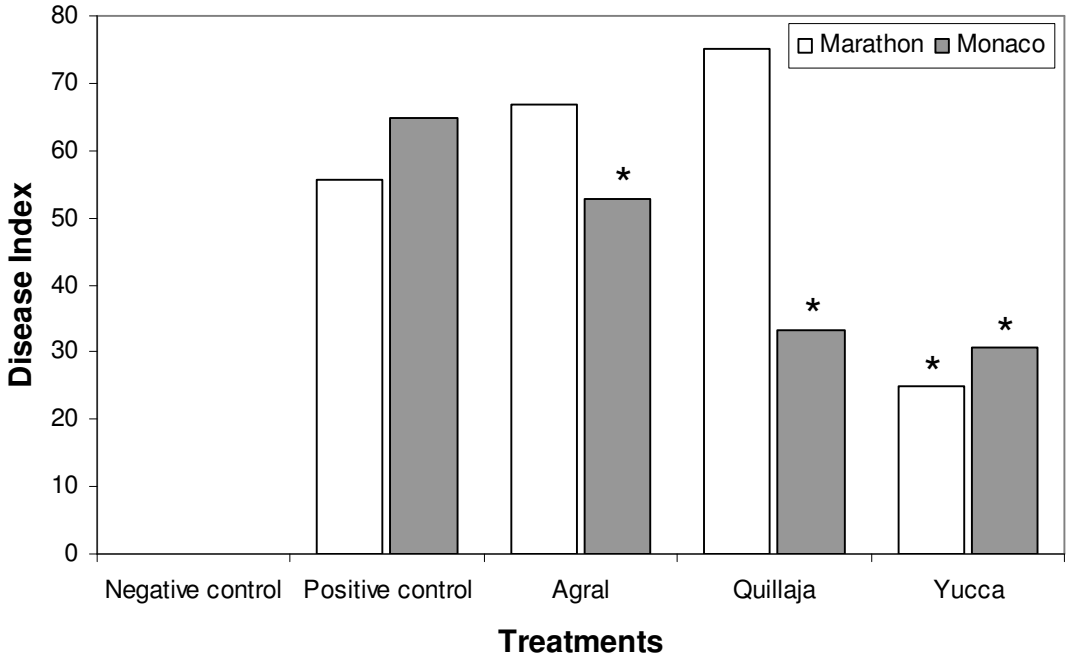

Figure 3.2. Effect of surfactants on clubroot severity: cv. Marathon and Monaco (2004)
***P<0.05**

Biocontrol agents: initial experiment 2004

Figure 3.3 shows the effectiveness of biocontrol treatments at controlling clubroot in the two calabrese cultivars. The two Bactolife products and the chitin product (bio-processed prawn shells) significantly reduced disease in both cultivars. The *P.*

chlororaphis treatment significantly reduced disease in cultivar Monaco but not cultivar Marathon. *Pseudomonas fluorescens* had no significant effect on disease in either cultivar. None of the biocontrol agents had a significant effect on plant fresh weight in cultivar Marathon but both of the Bactolife products significantly reduced fresh weight in cultivar Monaco (Appendix 1, Table 6 shows the effect of the biocontrol agents on plant fresh weight). The roots of the plants exposed to the Bactolife treatments did not grow out past the module compost.

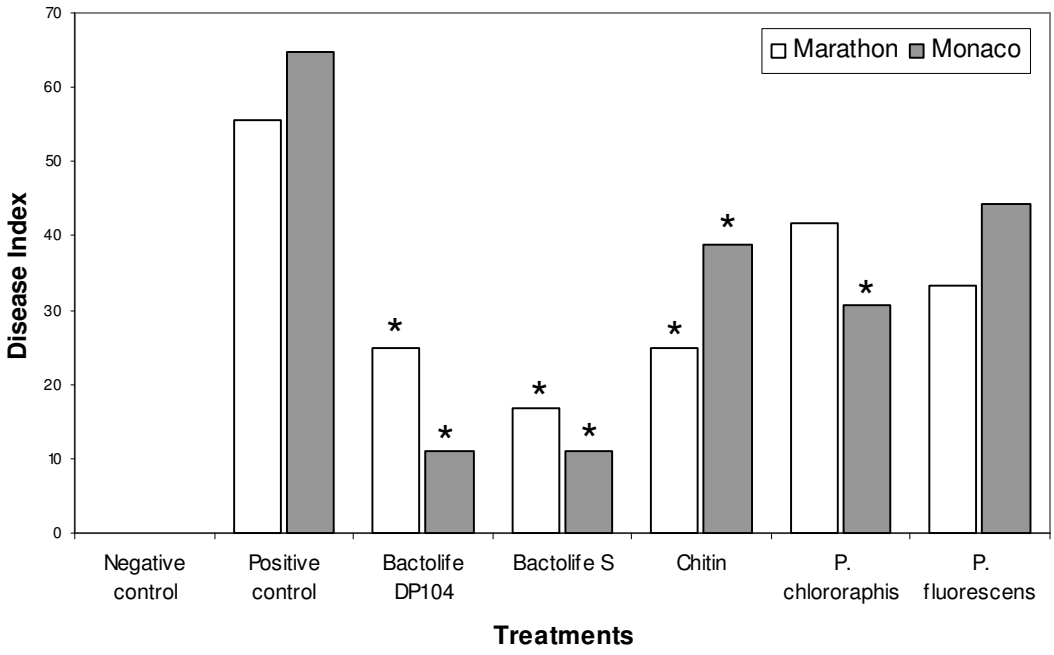

Figure 3.3. Effect of biocontrol agents on clubroot severity: cv. Marathon and Monaco (2004) *P<0.05

Calcium containing compounds: initial experiment 2004

The calcium treatments did not control disease in the cultivar Marathon (Figure 3.4). Calcium silicate was the only calcium treatment to give a significant increase in plant fresh weight compared with the positive control in this cultivar (Appendix 1, Table 8).

All of the calcium treatments with the exception of the gravel form of calcium oxide significantly reduced disease in cultivar Monaco. None of the treatments in table 3.4 had a significant effect on fresh weight in cultivar Monaco (Appendix 1, Table 8).

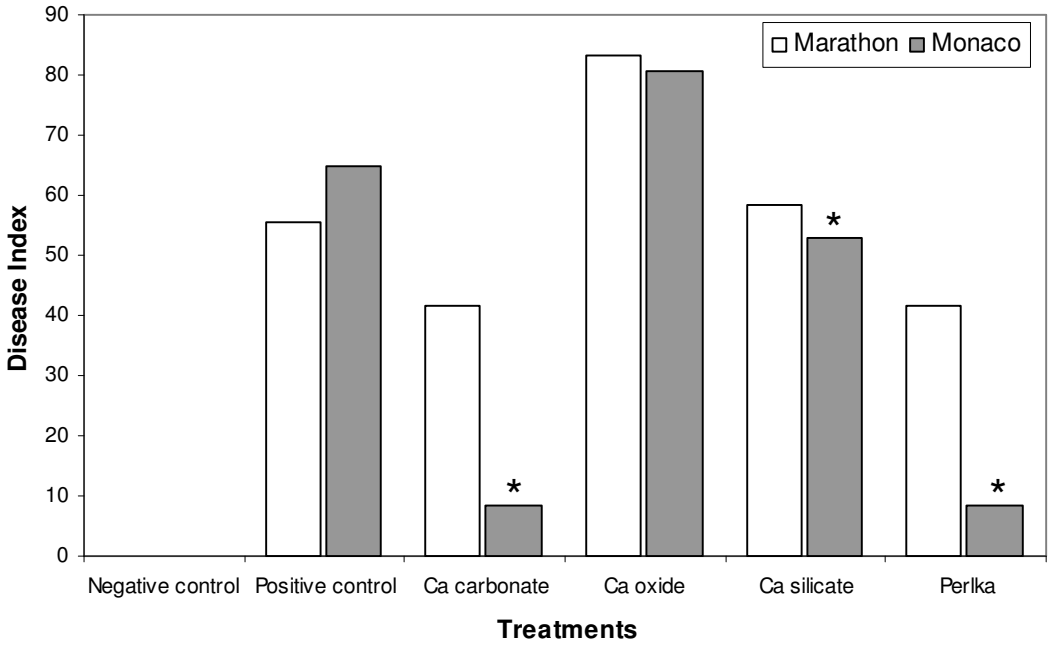

Figure 3.4. Effect of calcium containing compounds on clubroot severity: cv. Marathon and Monaco (2004) *P<0.05

Fungicides: initial experiment 2004

The effectiveness of fungicides at controlling clubroot disease in the two cultivars of calabrese can be seen in Figure 3.5. Ranman and Shirlan both completely controlled disease but did not have a significant effect on plant fresh weight in cultivar Marathon. The two fungicides tested also significantly reduced disease in cultivar Monaco but did not have a significant effect on plant fresh weight in this cultivar (Appendix 1, Table 10).

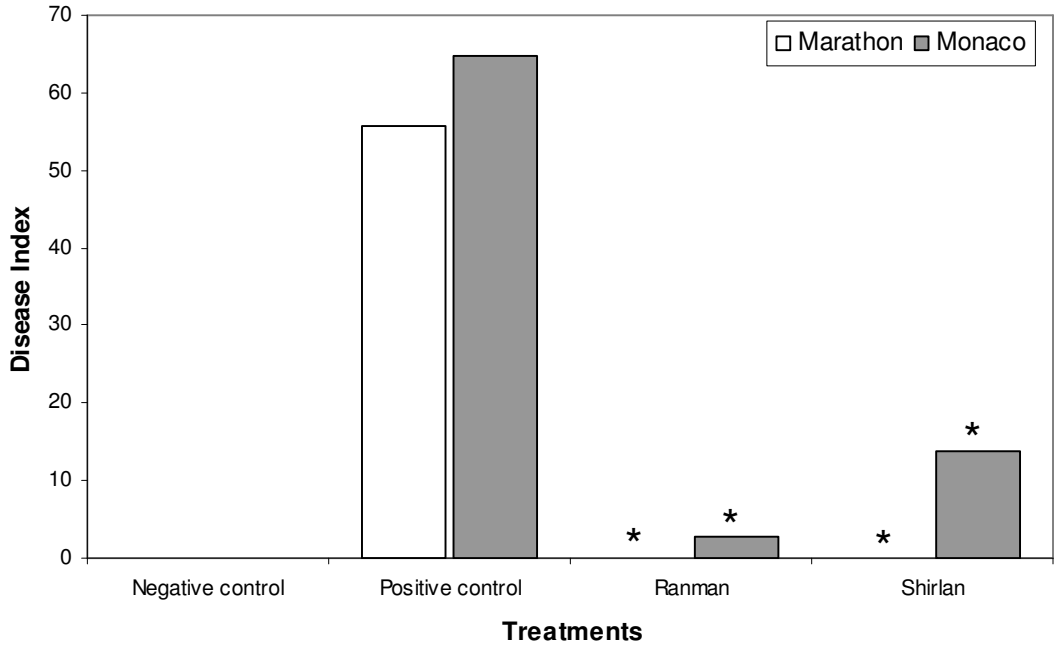

Figure 3.5. Effect of fungicides on clubroot severity: cv. Marathon and Monaco (2004) *P<0.05

General summary of initial glasshouse results 2004

Overall, the 2004 experiment showed that more treatments were effective at controlling disease in the cultivar Monaco than in the cultivar Marathon. The only treatments not to reduce disease in the Monaco cultivar of plants were the gravel form of calcium oxide, potassium tetraborate and the combination of biohumate and potassium tetraborate. The treatments that significantly reduced disease in both cultivars were the fungicides, the Bactolife products, crushed prawn shells, Yucca extract, and the combination of Biohumate + calcium carbonate and the combination of potassium tetraborate + calcium carbonate. The surfactants and the calcium containing compounds were effective at controlling disease in the Monaco cultivar but not the Marathon cultivar. The Biohumate treatment was the only treatment to significantly increase the disease level in the plants. This was seen in the cultivar Marathon experiment.

The Biohumate + calcium carbonate treatment and both of the Bactolife treatments significantly reduced plant fresh weight compared with the control plants in the Monaco cultivar. None of the treatments significantly reduced plant fresh weight in the cultivar Marathon but the calcium silicate treatment and the potassium tetraborate + calcium carbonate combination were the only treatments to significantly increased plant fresh weight.

Effectiveness of treatments at controlling clubroot in calabrese cv. Monaco – effects of timing, new dose rates and treatment combinations (2005)

From the 2004 experiment it was observed that plants that had very different amounts of diseased root tissue could still be classed within the same grade on the scale of 0-3. Therefore, the percentage of diseased roots compared with healthy roots on a plant system was used in place of the disease index calculation in experiments from 2005 onwards. The fresh weight of the diseased roots was divided by the total fresh weight of the whole root system to give the % disease figure. This method of disease measurement also allowed a standard error of means (SEM) calculation to be made on the variation associated with the mean amount of disease in the roots.

Biocontrol agents: 2005 experiment

The biocontrol agents were generally not effective at controlling disease in the 2005 experiment and therefore the results are reported in Appendix 1, Table 11. Bactolife DP104 gave better control than Bactolife S. Lower levels of *P. chlororaphis* gave slightly better control than higher levels although there was not a significant difference. Lower levels of *P. fluorescens* also gave slightly better control than higher levels.

The only treatments to significantly reduce disease level were the shell sand and spent mushroom compost at 30 % treatments (Figure 3.6). None of the biocontrol treatments had a significant effect on plant fresh weight (Appendix 1, table 11).

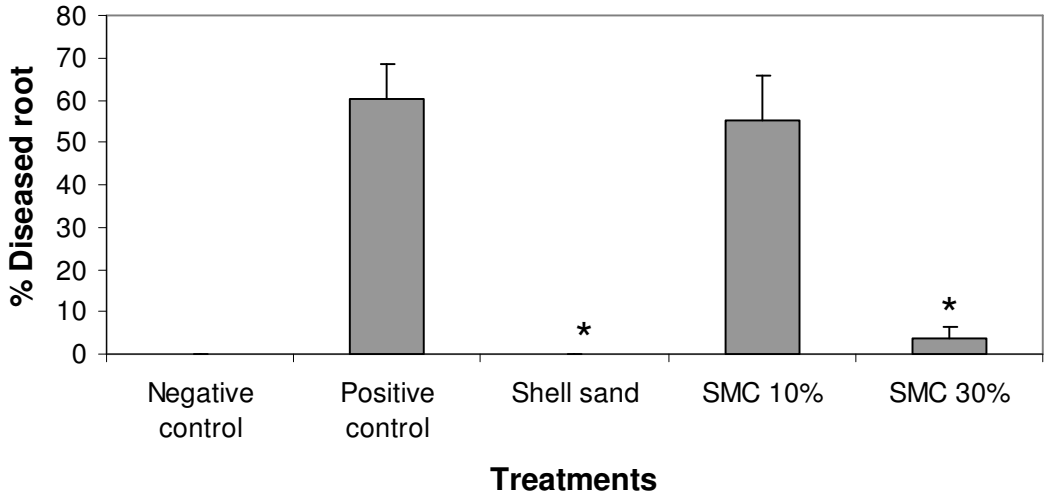

Figure 3.6. The effect of shell sand and SMC on clubroot: cv. Monaco (2005); *P<0.05, bars indicate SEM's.

Plant extracts: 2005 experiment

Table 12 in Appendix 1 shows that none of the plant extract treatments such as peppermint oil, rhubarb leaves or seaweed extract significantly controlled disease and neither did the liquid chitosan treatment. None of these treatments had a significant effect on plant fresh weight compared to the positive controls.

Combinations of Borax, calcium carbonate and Perlka: 2005 experiment

The Borax treatment significantly controlled disease in the 2005 experiment (Figure 3.7). The Borax and calcium carbonate combination of treatments also significantly controlled disease and was more effective at controlling disease than when either treatment was used on its own. The Borax + calcium carbonate + Perlka treatment completely controlled clubroot disease but it also caused the plants to die (Appendix 1, Table 13).

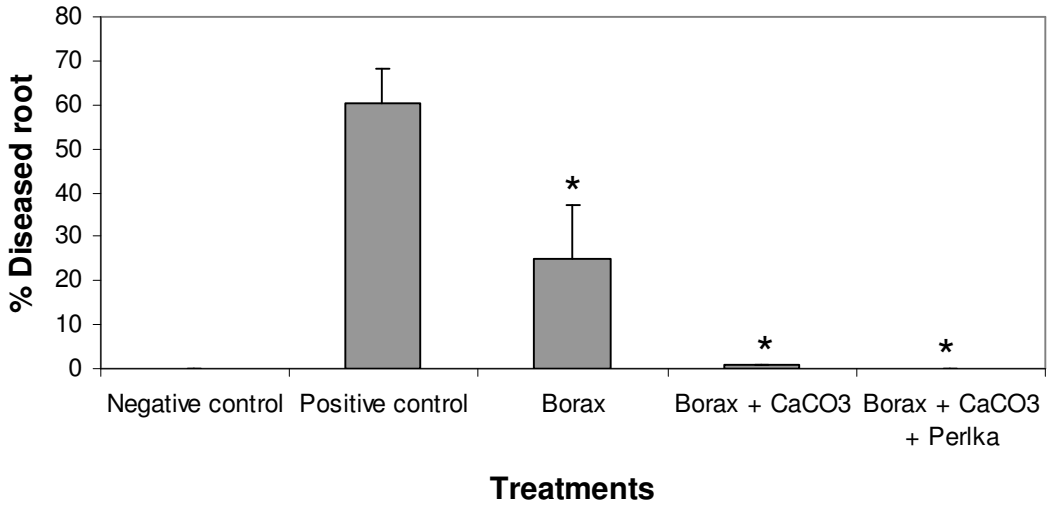

Figure 3.7 The effect of combinations of Borax, calcium carbonate and Perlka on clubroot: cv. Monaco (2005);*P<0.05, bars indicate SEM's.

Lime and Perlka treatments: 2005

The LimeX70 and powdered calcium oxide treatments completely controlled disease in the 2005 experiment (Figure 3.8). The calcium carbonate treatment also significantly controlled disease but did not control it completely. The Perlka treatment and the treatments that combined a lime and Perlka all completely controlled disease. This was because plants exposed to Perlka treatments died. This is why the Perlka treatments were observed to cause a significant reduction in plant fresh weight (Appendix 1, Table 14). The Perlka had been applied to the soil only one day in advance of transplanting.

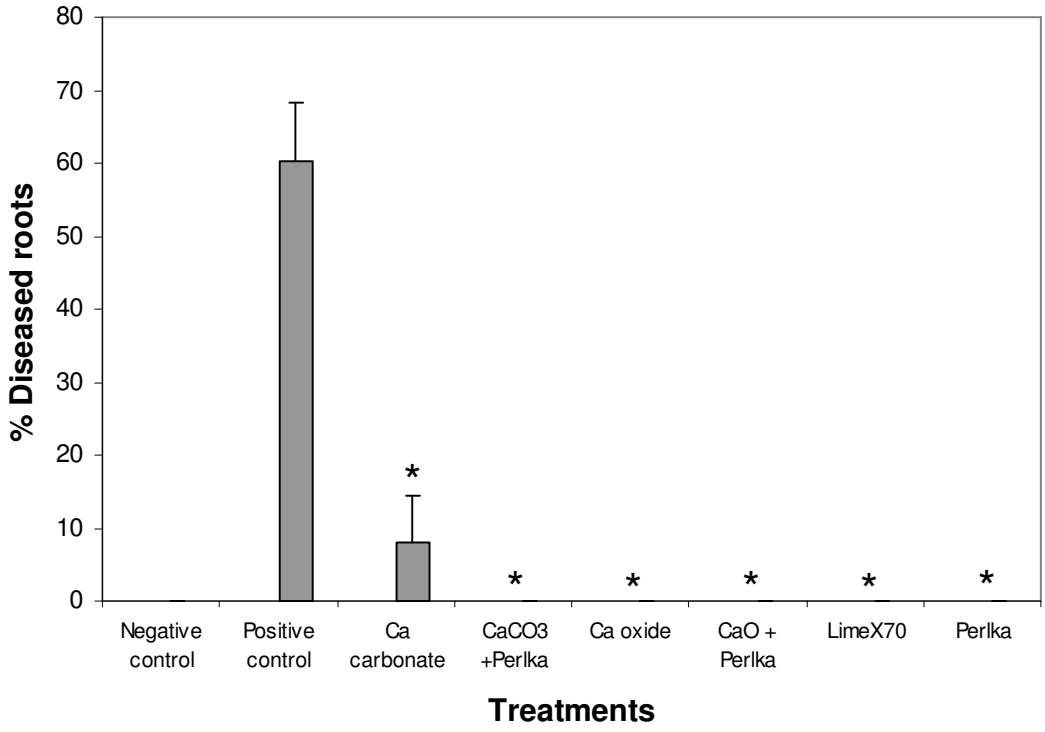

Figure 3.8. The effect of powdered limes and Perlka on clubroot: cv. Monaco (2005);*P<0.05, bars indicate SEM's.

Surfactants: 2005 experiment

The natural surfactants tested in the 2005 experiment were not effective at controlling disease and did not have any significant effect on plant fresh weight (Appendix 1, Table 15).

Fungicides: 2005 experiment

The fungicides Ranman and Shirlan significantly reduced the level of disease in the plants and Shirlan controlled it completely (Figure 3.9). These treatments did not have a significant effect on plant fresh weight (Appendix 1, Table 16).

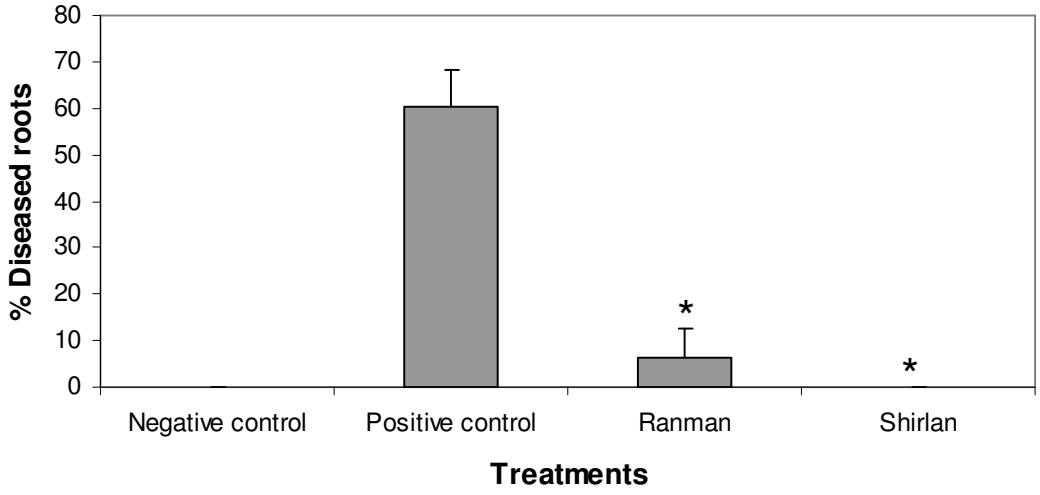

Figure 3.9. Effect of fungicides on clubroot: cv. Monaco (2005);* P<0.05, bars indicate SEM's.

Effectiveness of treatments at controlling clubroot in calabrese cv. Monaco – effects of timing of treatment addition and more treatment combinations(2006)

The percentage of the roots that were diseased in the positive control plants in the 2006 experiment was very low compared to positive control plants in past experiments. The reason for this was unclear. This spore inoculum had been passed through many rounds of infection of Chinese cabbage in creating the large amount of spores to be used in this experiment.

Combinations of Amistar and *P. chlororaphis*: 2006

The fungicide Amistar increased the level of disease seen in the plants (Figure 3.10). The amount of diseased tissue on the roots was much greater than in the positive control plants. Amistar was slightly less effective at controlling disease when it was applied along with *Pseudomonas chlororaphis* than when it was applied on its own but not significantly.

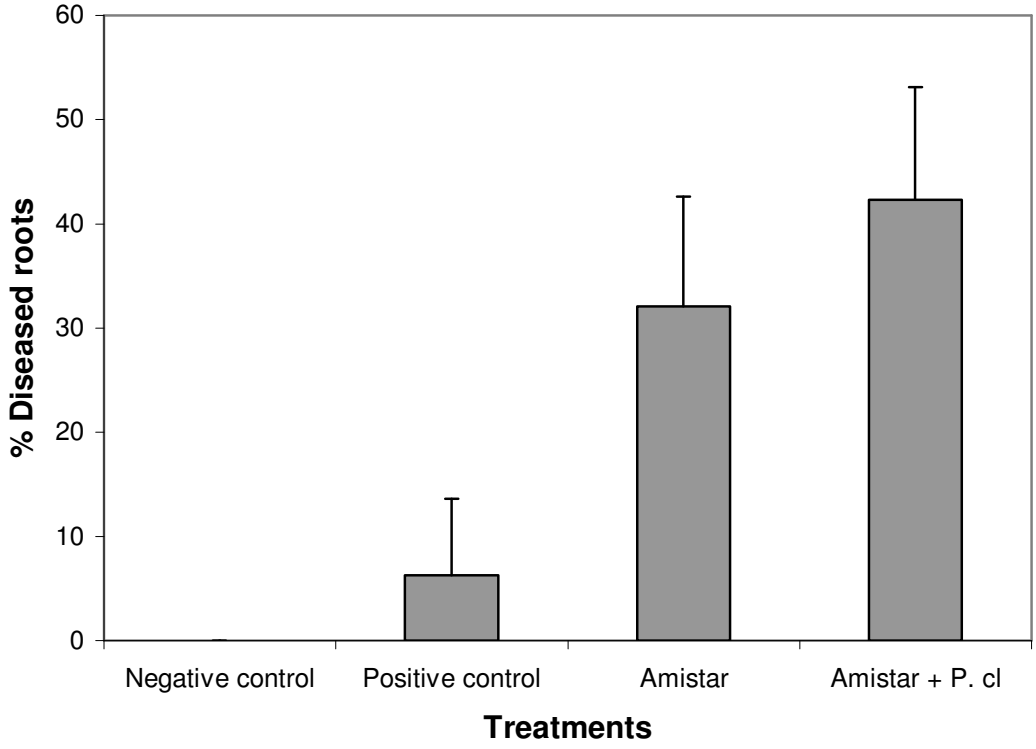

Figure 3.10. Effect of Amistar and *P. chlororaphis* on clubroot: cv. Monaco (2006); * P<0.05, bars indicate SEM's.

Biocontrol agents: 2006

The biocontrol agents did not significantly reduce disease (Appendix 1, Table 18). They also had no significant effect on plant fresh weight. Spent mushroom compost was slightly more effective at controlling disease when *P. chlororaphis* was added to the soil with it than when it was added to the soil on its own but not significantly. Spent mushroom compost raised the final pH of the soil to above pH 7 (Appendix 1, Table 18). Plants exposed to *P. chlororaphis* had woody and abnormal tap roots compared with the control plants.

Nutritional treatments: 2006

None of the nutritional compounds added to the soil in the 2006 experiment (Bod Ayre seaweed, calcium nitrate, copper carbonate and gypsum) had a significant effect on

disease severity. Calcium nitrate gave the most significant increase in plant fresh weight compared with the positive controls in the 2006 experiment (Appendix 1, Table 19). The main root and secondary root system of plants exposed to this treatment were equally infected by clubroot disease. The copper carbonate treatment made the taproot look abnormal and woody compared to the control plants.

Borax, calcium carbonate and *P. chlororaphis*: 2006

Borax had no significant effect on plant fresh weight or disease control (Figure 3.11). It increased soil pH to pH 7. Plants exposed to this treatment did not form large galls but the fibrous roots had high levels of diseased tissue. When calcium carbonate was used along with Borax, the disease level was significantly reduced. When Borax, calcium carbonate and *P. chlororaphis* were used in combination, disease was completely inhibited. Borax was less effective at controlling disease when added to the soil along with *P. chlororaphis*. None of the treatments had a significant effect on plant fresh weight (Appendix 1, Table 20).

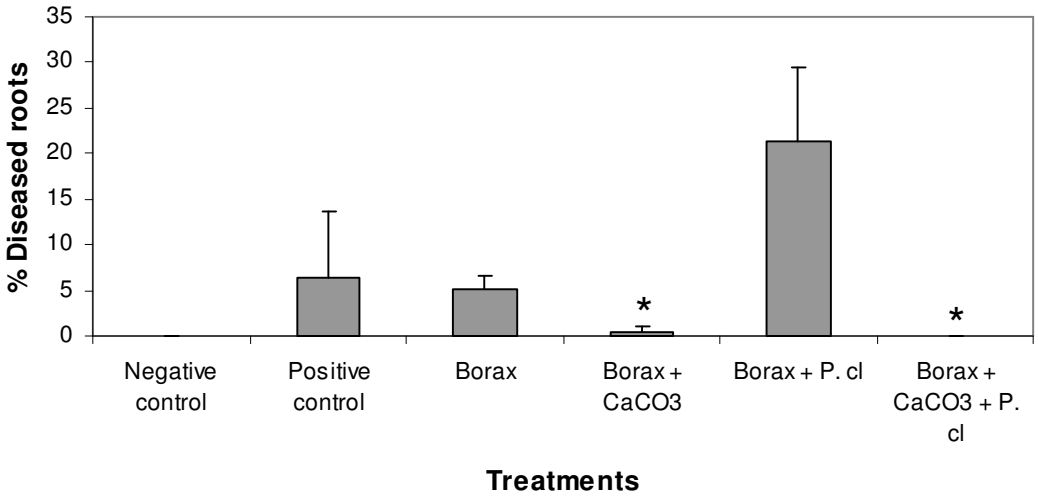

Figure 3.11 Effect of combinations of Borax calcium carbonate and *P. chlororaphis* on clubroot: cv. Monaco (2006); * P<0.05, bars indicate SEM's.

Combinations of calcium carbonate, Amistar and *P. chlororaphis*: 2006

The calcium carbonate treatment significantly reduced the level of disease but had no effect on plant fresh weight (Figure 3.12). When *P. chlororaphis* was added to the soil along with calcium carbonate, the controlling effect of the calcium carbonate was reduced. Adding both *P. chlororaphis* and Amistar along with calcium carbonate also reduced the controlling effect of calcium carbonate but gave slightly better control than when calcium carbonate and *P. chlororaphis* were added to the soil together. The combination of calcium carbonate + powdered calcium oxide completely controlled disease. The combination had no significant effect on plant fresh weight (Appendix 1, Table 21).

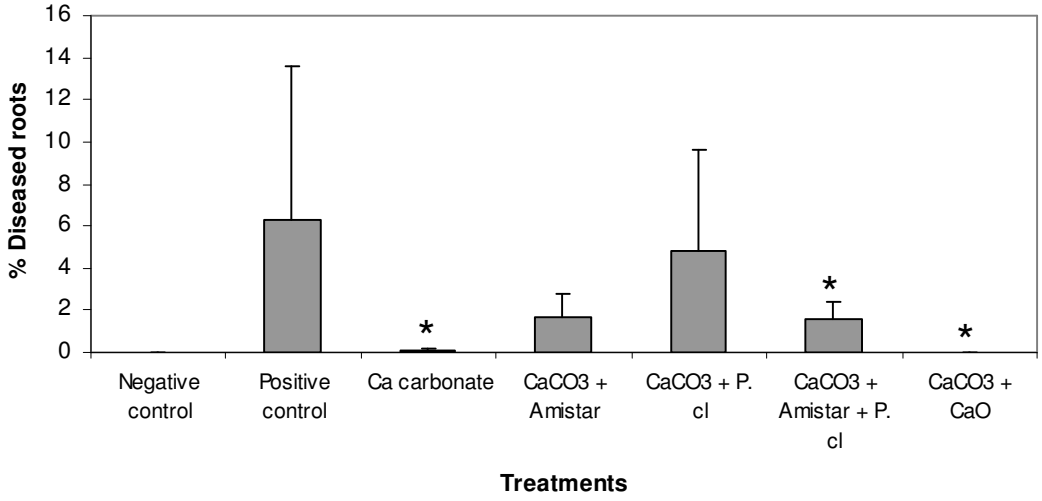

Figure 3.12. Effect of combinations of calcium carbonate, Amistar and *P. chlororaphis* at controlling clubroot: cv. Monaco (2006); * P<0.05, bars indicate SEM's.

Combinations of calcium oxide, Shirlan and *P. chlororaphis*: 2006

Figure 3.13 shows that the powdered calcium oxide completely inhibited disease compared with the controls. Adding Shirlan along with calcium oxide also completely inhibited disease. When *P. chlororaphis* was added to the soil along with calcium oxide, some disease was seen on the roots. The tap root appeared to be shorter on plants

exposed to *P. chlororaphis* than in the control plants. The combination of calcium oxide, Shirlan and *P. chlororaphis* also completely inhibited disease.

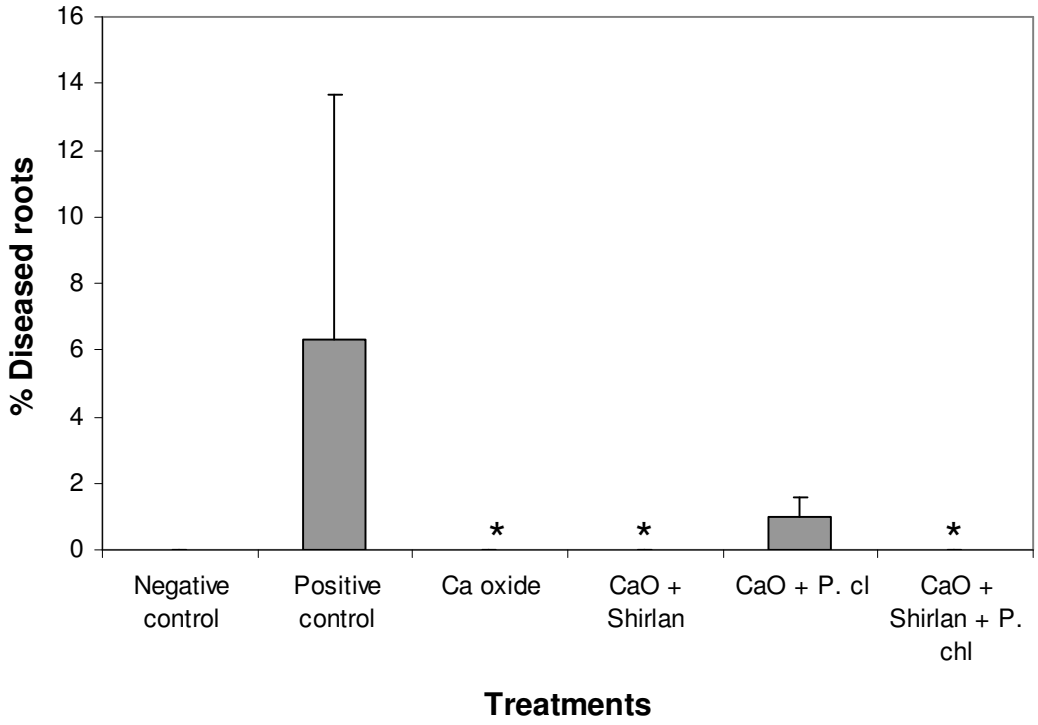

Figure 3.13. Effect of combinations of calcium oxide, Shirlan and *P. chlororaphis* at controlling clubroot: cv. Monaco (2006); * P<0.05, bars indicate SEM's.

Combinations of LimeX, Shirlan, and P .chlororaphis: 2006

All plants exposed to LimeX 70 showed no sign of disease even when other treatments were added in combination (Appendix 1, Table 23). Adding Shirlan and *P. chlororaphis* along with LimeX70 increased the fresh weight of the plants slightly compared to the controls but this was not a significant increase (Appendix 1, Table 23).

Perlka and QuickCal: 2006

Figure 3.14 shows the effect of adding Perlka and QuickCal to the soil. The Perlka treatment on its own reduced disease but not significantly compared to the controls. The disease was mostly confined to the fibrous roots of plants exposed to this treatment.

This treatment significantly increased the fresh weight of the plants (Appendix 1, Table 24). The combination of Perlka + QuickCal completely inhibited disease and also significantly increased plant fresh weight. The QuickCal treatment on its own did not significantly reduce disease or have an effect on plant fresh weight (Appendix 1, Table 24). The main root system of plants exposed to QuickCal showed disease.

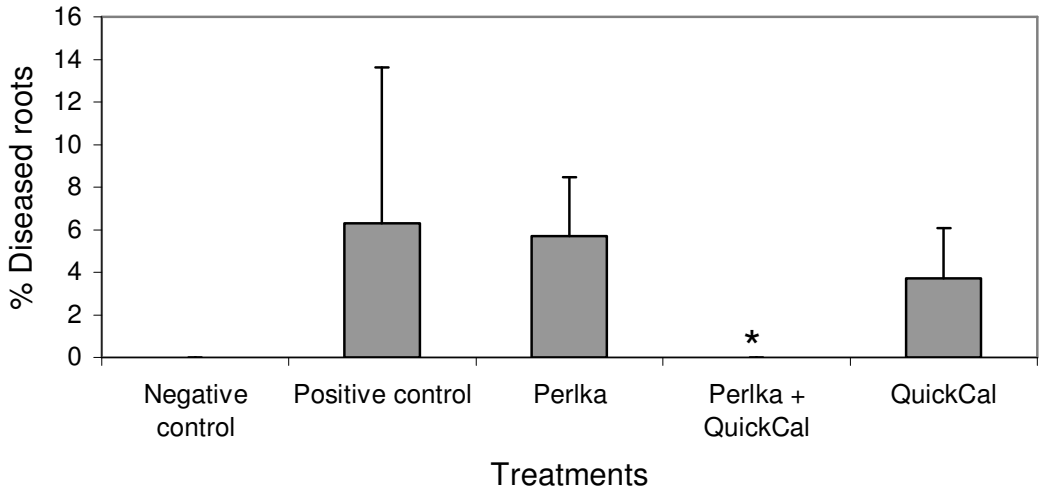

Figure 3.14. Effect of combinations of Perlka and QuickCal on clubroot: cv. Monaco (2006); * P<0.05, bars indicate SEM's.

Peppermint, SIPeco and Softguard: 2006

The peppermint oil treatment had no significant effect on plant fresh weight and increased the percentage of disease on the roots compared to the control plants but this was not significant according to the analysis used (Figure 3.15). The tap root of plants exposed to this treatment also looked abnormal compared with the control plants; they were woody and short and had sparse lateral roots. When powdered calcium carbonate was added along with peppermint oil, disease was completely inhibited (Figure 3.15). The SIPeco treatment based on hydrogen peroxide had no significant effect on plant fresh weight (Appendix 1, Table 25). The roots exposed to this treatment had high levels of diseased tissue – more than was seen in the positive controls. The Softguard

liquid chitosan treatment had no significant effect on plant fresh weight or on controlling disease (Figure 3.15).

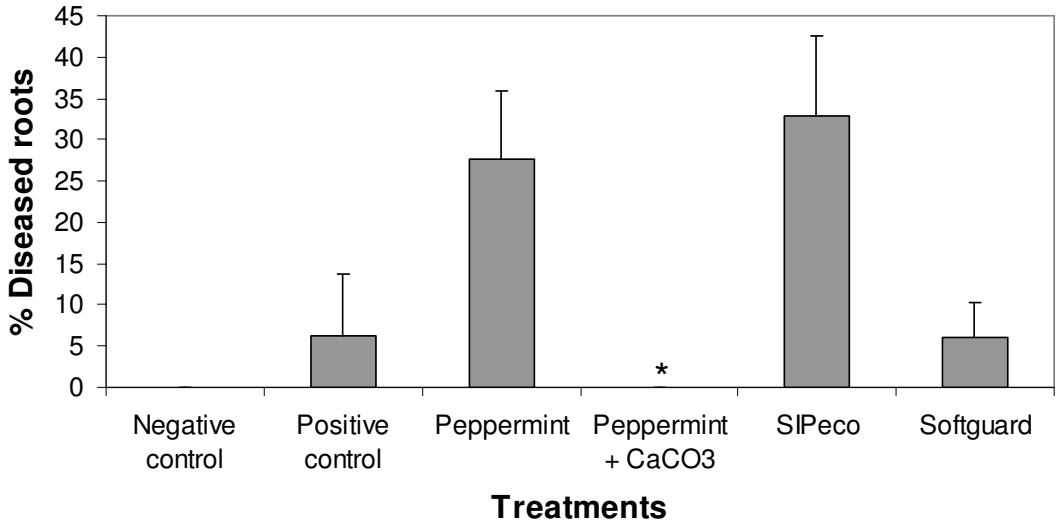

Figure 3.15. Effect of calcium carbonate, Peppermint, SIPeco and Softguard on clubroot: cv. Monaco (2006); * P<0.05, bars indicate SEM's.

Combinations of Quillaja, calcium carbonate and *P. chlororaphis*: 2006

The Quillaja treatment did not have a significant effect on plant fresh weight, disease level or soil pH but it did cause a higher percentage of roots to be diseased compared to the control plants (Figure 3.16). When calcium carbonate was added along with Quillaja, the disease level was reduced compared to if Quillaja was used on its own, but this combination was not as effective at controlling disease as when calcium carbonate was used on its own. When *P. chlororaphis* was added along with Quillaja and calcium carbonate, the level of disease increased compared to if only the Quillaja and calcium carbonate had been used, but this was not significant. No treatment in combination with Quillaja had any significant effect on disease or fresh weight (Appendix 1, Table 26).

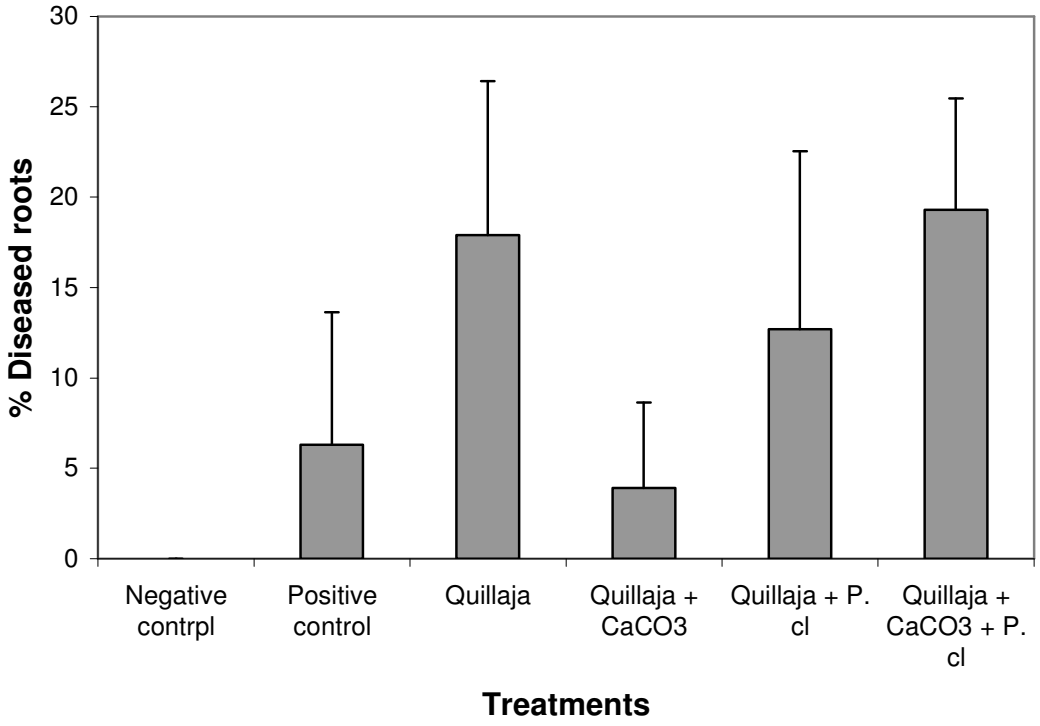

Figure 3.16. Effect of combinations of Quillaja, calcium carbonate and *P. chlororaphis* on clubroot: cv. Monaco (2006); * P<0.05, bars indicate SEM's.

Combinations of shell sand, Shirlan and *P. chlororaphis*

The crushed whelk and scallop shells (shell sand) had no significant effect on plant fresh weight. It reduced the disease level but this was not significant to the P<0.05 level (Figure 3.17). Adding Shirlan or Shirlan + *P. chlororaphis* along with the shell sand gave no significant increase in disease control than if shell sand was added on its own. When *P. chlororaphis* was added along with shell sand, the disease was completely inhibited. This was one of the few occasions when adding *P. chlororaphis* in combination with another treatment gave a reduction in disease severity. It was common for plants exposed to shell sand to only have one infected root – the first main lateral root was usually very swollen when exposed to this treatment.

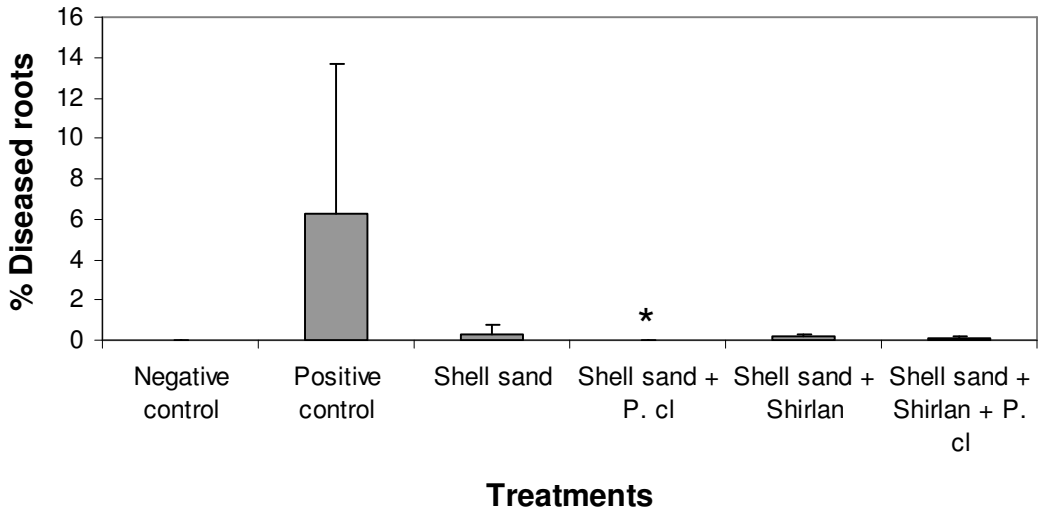

Figure 3.17. Effect of combinations of shell sand, Shirlan and *P. chlororaphis* on clubroot: cv. Monaco (2006); * P<0.05, bars indicate SEM's.

Combinations of Shirlan, calcium carbonate and P. chlororaphis

Figure 3.18 shows that Shirlan on its own significantly controlled disease but it had no significant effect on plant fresh weight (Appendix 1, Table 28). Shirlan used in combination with calcium carbonate completely controlled disease. If P. chlororaphis was used in combination with Shirlan, slight disease was seen on the roots. All combinations of Shirlan with calcium carbonate and P. chlororaphis significantly reduced disease compared to the positive control plants but had no significant effect on plant fresh weight (Appendix 1, Table 28).

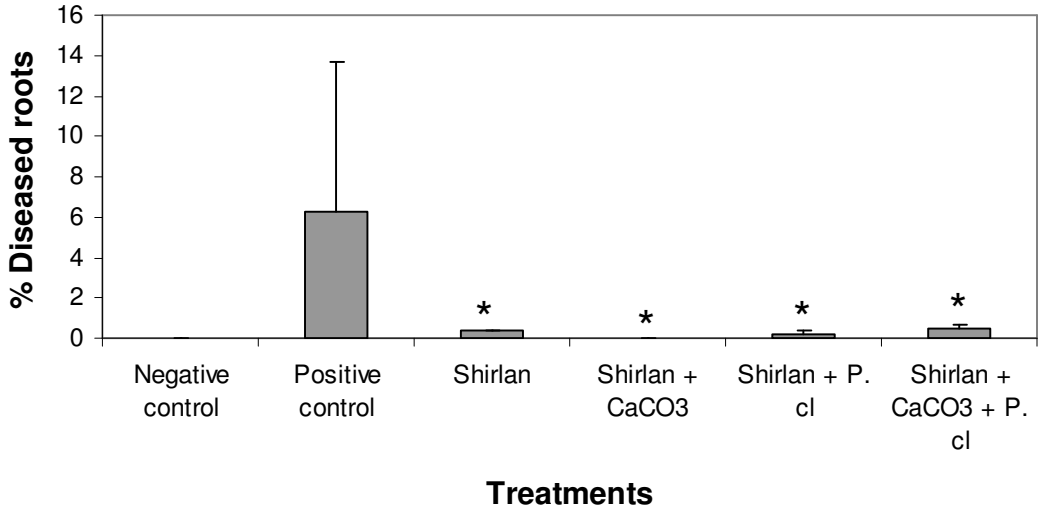

Figure 3.18. Effect of combinations of, Shirlan, calcium carbonate and *P. chlororaphis* on clubroot: cv. Monaco (2006); * P<0.05, bars indicate SEM's.

The initial pH of the John Innes compost prior to treatments was pH 5.7. The control treatment pots therefore show that the plants had increased the pH level of the soil to pH 6.6 (Table 3. 10). Treatments that significantly increased the final soil pH compared to the control plants can be seen in table 3.9.

Table 3.9. Final soil pH of treatments that significantly affected soil pH compared with controls (2006)

Treatment	Final soil pH	Treatment	Final soil pH
Negative control	6.6	LimeX	7.4
Positive control	6.6	LimeX + Shirlan	7.5
SMC 30 %	7.1	LimeX + *P. chlororaphis*	7.4
Borax + $CaCO_3$	7.4	LimeX + Shirlan + *P. chlororaphis*	7.6
Borax + $CaCO_3$ + *P. chlororaphis*	7.3	Peppermint + $CaCO_3$	7.5
Ca carbonate	7.1	Perlka + QuickCal	8.9
$CaCO_3$ + Amistar	7.3	QuickCal	7.4
$CaCO_3$ + *P. chlororaphis*	7.3	Quillaja + $CaCO_3$	7.6
$CaCO_3$ + Amistar + *P. chlororaphis*	7.3	Quillaja + $CaCO_3$ + *P. chlororaphis*	7.1
$CaCO_3$ + CaO	7.4	Shell sand	7.3
Ca oxide	7.7	Shell sand + *P. chlororaphis*	7.3

CaO + Shirlan	7.5	Shell sand + Shirlan + *P. chlororaphis*	7.4
CaO + *P. chlororaphis*	7.4	Shirlan + $CaCO_3$	7.3
CaO + Shirlan + *P. chlororaphis*	7.3	Shirlan + $CaCO_3$ + *P. chlororaphis*	7.4

The extractable calcium content of the soil that had been exposed to selected calcium containing treatments was analysed when the plants were harvested after six weeks of exposure to the treatments. The results of this analysis can be seen in table 3.10.

Table 3.10. The disease index, final soil pH and final soil extractable calcium content of soil exposed to selected calcium containing treatments (2006)

Treatment	Amount of product applied (t/ha)	Disease Index	pH	Extractable calcium (mg/l)
John Innes No. 1 compost			5.7	2640
Positive control		41.7	6.6	2200
Calcium carbonate	1.5	2.8	7.1	7850
Calcium carbonate + calcium oxide	1.5 + 0.75	0	7.4	7370
Calcium nitrate	0.25	50	6.2	2690
Calcium oxide	0.75	0	7.7	5950
Calcium oxide + P. chlororaphis	0.75	8.3	7.4	3560
Gypsum	2	33.3	6.3	3870
LimeX	4	0	7.4	6760
Perlka	0.20	16.7	6.9	2910
Perlka + QuickCal	0.20 + 0.75	0	8.9	6160
QuickCal	0.75	22.2	7.4	5300
Quillaja + Calcium carbonate	40 l + 1.5	16.7	7.6	6710
Shell sand	5	5.6	7.3	13900
Spent mushroom compost	30	8.3	7.1	4520

The extractable calcium results show that the positive control plants had consumed 440 mg/l of calcium over the six weeks of the experiment. The treatments that gave total control of disease had an extractable calcium content in the soil of 5950 mg/l or more. However, treatments that increased the soil calcium to above this level did not always completely inhibit disease. The calcium nitrate and Perlka treatments did not increase the soil calcium content. The shell sand treatment gave the highest increase in soil

calcium content out of all of the treatments analysed. There was less calcium in the soil when calcium oxide was added to the soil with *P. chlororaphis* than when it was added on its own. There was less calcium in the soil when Quillaja was added to the soil along with calcium carbonate than when calcium carbonate was added to the soil on its own. The combination of calcium carbonate and calcium oxide had a lower final soil extractable calcium level than when calcium carbonate was added on its own but a higher level than when calcium oxide was added on its own. The combination of Perlka and Quickcal had an increased final soil calcium level than when either of the two treatments were used on their own. Treatments that caused the same final soil pH did not have the same final calcium level.

Overall summary of effect on fresh weight of treatments in the 2006 experiment

Only Perlka, Perlka + QuickCal and calcium nitrate gave an increase in plant fresh weight. No treatment significantly reduced plant fresh weight.

Overall summary of effect on disease treatments in the 2006 experiment

Amistar, peppermint oil, Borax + *P. chlororaphis*, and SIPeco slightly increased the disease seen on the roots. Treatments containing calcium carbonate, Perlka, powdered calcium oxide, shell sand, LimeX, *P. chlororaphis*, Shirlan and spent mushroom compost significantly reduced disease. Table 3.11 gives the treatments that completely inhibited disease.

Observations on soil pH

The pH range of the soil after having the treatments added was from pH 6.2 – 8.9. Perlka added with Quickcal showed the biggest increase in soil pH reaching a pH of 8.9. Treatments which completely inhibited disease increased the pH to above pH 7.3 but soil that had been raised to above pH 7.3 by some treatments such as Quillaja + calcium carbonate still showed signs of clubroot disease.

Calcium levels

There was no level of calcium in the soil that could be said to have a complete controlling effect on disease if this level was reached.

Table 3.11. Treatments and treatment combinations that completely inhibited the development of clubroot disease on calabrese cv. Monaco plants in 2006; soil pH, extractable calcium and fresh weight results

Treatment	Soil pH	Soil extractable calcium (mg/l)	Fresh weight (g); SEM
Borax + CaCO3 + P. chlororaphis	7.3	*	13.72 ± 2.54
CaCO3 + CaO	7.4	7370	17.06 ± 1.66
CaO	7.7	5950	17.00 ± 2.69
CaO + Shirlan	7.5	*	16.18 ± 2.13
CaO + Shirlan + P. chlororaphis	7.3	*	16.84 ± 3.65
LimeX	7.4	6760	18.05 ± 2.24
LimeX + Shirlan	7.5	*	18.06 ± 3.88
LimeX + P. chlororaphis	7.4	*	16.25 ± 3.58
LimeX + Shirlan + P. chlororaphis	7.6	*	20.27 ± 4.64
Perlka + QuickCal	8.9	6160	25.13 ± 5.03
Peppermint + CaCO3	7.5	*	15.55 ± 2.05
Shell sand + P. chlororaphis	7.3	*	15.59 ± 3.21
Shirlan + CaCO3	7.3	*	16.83 ± 2.78

* extractable calcium measurements were not made for these treatments

General comments

Treatments containing LimeX gave the best results for disease control. The most effective treatment was Perlka + Quickcal as this was the only treatment to give a significant increase in fresh weight, total inhibition of disease and a significant increase in soil pH. Most other treatments, when added in combination, did not reduce disease level any more than when individual treatments were used. When *P. chlororaphis* was

added in combination with other treatments, it caused the disease level to increase compared to if the other treatments were used on their own. The only cases where it had an additive effect at disease control was when it was added along with shell sand or Amistar or with the combination of Borax + calcium carbonate.

Oilseed rape meal - timing of addition experiment

Oilseed rape meal was added to inoculated pots at a number of weeks before the plants were transplanted into the pots. This was to determine if adding it at different time periods before transplanting could reduce final disease levels. The results of this experiment can be seen in Figure 3.19 and in Appendix 1, Table 29.

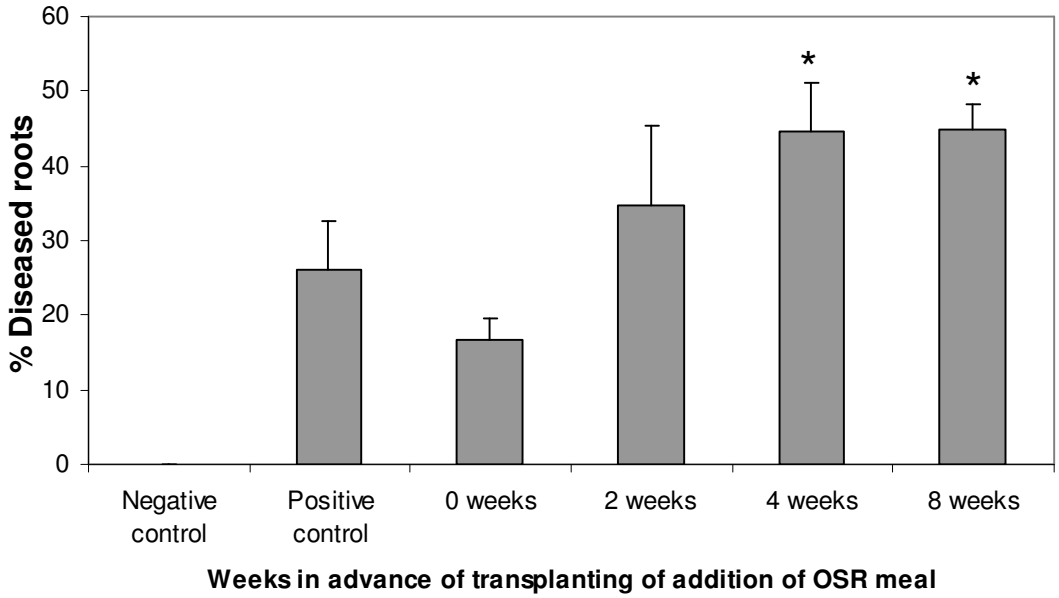

Figure 3.19. Effect of adding oilseed rape meal 8, 4, and 2 weeks before and at transplanting on clubroot: cv. Monaco (2006); * P<0.05, bars indicate SEM's.

The results show that the further in advance of transplanting the oilseed rape meal was added to the soil, the higher the disease level on the roots after six weeks. Adding the oilseed rape meal to the pots six and eight weeks before transplanting significantly

increased the percentage of disease on the roots compared to the positive control plants which had no added oilseed rape meal. The most effective time point for controlling disease was to add the oilseed rape meal to the pots at transplanting but this still did not give a significant reduction in disease level. Adding oilseed rape meal to the pots no matter how far in advance of transplanting cased a significant increase in plant fresh weight compared to the positive control plants (Appendix 1, Table 29). Adding the oilseed rape meal to the soil four weeks before transplanting gave the best results for plant fresh weight. The timing of addition of the oilseed rape meal also had an effect on plant root weight. The further in advance of transplanting the oilseed rape meal was added to the pots, the greater the root weight of the plants after six weeks (Appendix 1, Table 29). Adding oilseed rape meal to the pots six weeks and eight weeks in advance of transplanting significantly increased the root weight of the plants. This corresponds to the weight of the diseased tissue.

2007 experiment – Effectiveness of treatments at controlling clubroot in autoclaved field soil

In the 2007 experiment, plants transplanted into the John Innes No. 2 soil inoculated with 10^6 spores/ml had a disease index of 100. The non-autoclaved field soil had a disease index of 92. No disease was seen in the positive control autoclaved field soil containing 10^4 spores/ml. Only one plant exposed to 10^6 spores/ml in the field soil showed disease, two plants exposed to 10^8 spores/ml in the field soil showed disease. These results are shown in table 3.12. None of the other treated soils had any plants that showed signs of disease. Table 3.13 shows the fresh weight and root weight results of the experiment. The positive control plants showed that by adding more clubroot spores to the soil, the fresh weight and root weight of the Chinese cabbage plants increased. Calcium carbonate + *P. chlororaphis* in soil that had 10^4 spores/ml added to it gave the most significant increase in fresh weight of the plants. *P. chlororaphis* was generally shown to have a positive effect on plant growth. Most treatments increased plant fresh

weight and root weight compared to the positive control containing 10^4 spores/g soil with the exception of calcium carbonate in soil with 10^4 spores/g soil added to it.

Table 3.12. Disease index of artificially inoculated compost, naturally infested field soil and autoclaved field soil re-inoculated with spores

Soil	Disease Index
John Innes No.2	100
Field soil	92
Autoclaved field soil	0
Re-inoculated autoclaved field soil 10^4 spores/g soil	0
Re-inoculated autoclaved field soil 10^6 spores/g soil	0.8
Re-inoculated autoclaved field soil 10^8 spores/g soil	1.7

Table 3.13. The effect of adding different clubroot spore loads and combinations of calcium carbonate and *P. chlororaphis* treatments to re-inoculated clubroot infested field soil that has been autoclaved on plant fresh weight and root weight

Treatment	Fresh weight (g); SEM	Fresh weight Sig. probability	Root weight (g); SEM	Root weight Sig. probability
Negative control	0.75 ± 0.104	0.11	0.210 ± 0.030	0.024*
Positive control 104spores/g soil	0.322 ± 0.040		0.058 ± 0.006	
Positive control 10^6 spores/g soil	0.478 ± 0.085	0.338	0.172 ± 0.026	0.083
Positive control 10^8 spores/g soil	0.914 ± 0.231	<0.001*	0.218 ± 0.049	0.021*
P. chlororaphis 10^4spores/g soil	0.716 ± 0.074	0.016*	0.255 ± 0.056	0.003*
P. chlororaphis 10^6 spores/g soil	0.970 ± 0.128	<0.001*	0.348 ± 0.066	<0.001*
CaCO3 10^4 spores/g soil	0.395 ± 0.067	0.656	0.147 ± 0.027	0.173
CaCO3 10^6 spores/g soil	0.658 ± 0.111	0.040*	0.280 ± 0.071	<0.001*
CaCO3 + *P. chlororaphis* 10^4 spores/g soil	1.248 ± 0.186	<0.001*	0.328 ± 0.049	<0.001*
CaCO3 + *P. chlororaphis* 10^6 spores/g soil	1.030 ± 0.085	<0.001*	0.383 ± 0.038	<0.001*

Compared to 10^4 positive control FW LSD = 0.335
Compared to 10^4 positive control RW LSD = 0.134

Adding treatments to the outside of module compost experiment

An experiment was carried out to determine if adding powdered treatments to the outside of the module compost could prevent zoospores from entering the main initial root zone. Adding some of the most effective treatments to the outside of the module compost showed that the powdered calcium oxide treatment was the only treatment that significantly reduced the level of disease in the plants (Figure 3.20). None of the treatments significantly increased the fresh weight of the plants. Calcium oxide treated plants have a high SEM for fresh weight because many plants exposed to this treatment died, but the plants that did survive were very large and healthy.

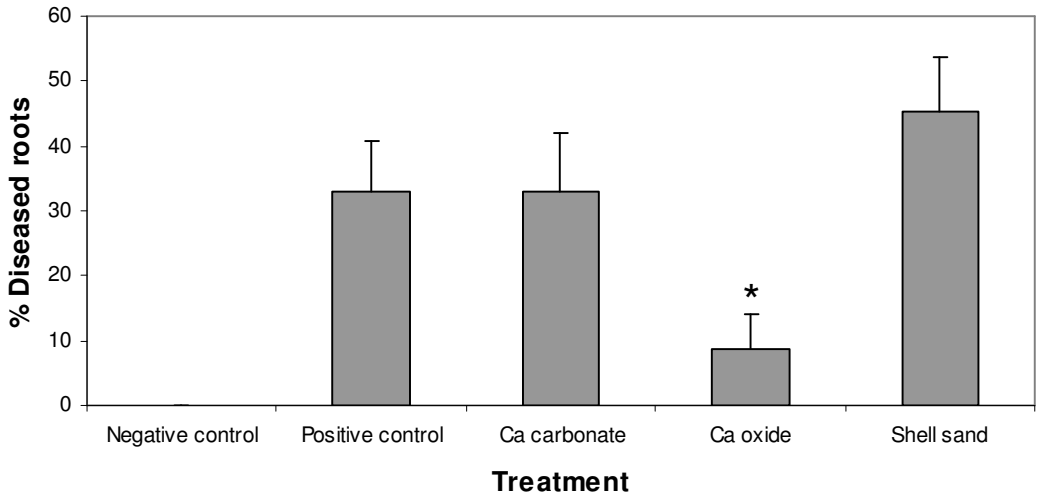

Figure 3.20 Effect of adding powdered treatments to outside of module compost on clubroot: cv. Monaco (2006); * P<0.05, bars indicate SEM's.

Discussion

The effectiveness of treatments at controlling clubroot disease in the glasshouse

Fungicides

Fungicides that were specific against oomycete fungi (i.e Ranman (cyazofamid) and Shirlan (fluazinam)) gave significant control against clubroot in these glasshouse experiments. The broad-spectrum fungicide Amistar significantly increased the level of disease on the roots. Possible explanations for these results are discussed below.

Ranman

In the 2004 experiments, the fungicide Ranman was the most effective treatment of those tested at controlling disease. It completely controlled clubroot in the cultivar Marathon and was the most effective treatment at reducing disease in the cultivar Monaco. Ranman was also very effective at controlling disease in the 2005 experiment although again, not completely. The disease did not progress beyond the initial root hair stage of the life-cycle of *P. brassicae* when treated with Ranman because clubbing of the tap root was never observed. Ranman was also the most effective treatment tested in 2005 for increasing plant fresh weight. The results indicate that Ranman can provide consistently good clubroot control in glasshouse conditions and is not phytotoxic because it did not cause a significant reduction in fresh weight or cause the plant to show any abnormalities in physiology.

Ranman contains the active ingredient cyazofamid (4-chloro-2-cyano-N, N-dimethyl-5-p-tolylimidazol-1-sulfonamide) which has been shown to have high levels of activity against Oomycetes and *P. brassicae* (Mitani *et al.,* 2003). Cyazofamid inhibits resting spore germination by inhibiting the electron transport in Oomycete fungal mitochondria resulting from binding at a specific site on cytochrome b (Mitani *et al.,* 2003). *P. brassicae* is not an Oomycete fungus but it suggests that the structural features of the

cytochrome bc1 complex may be similar between Oomycetes and *P. brassicae* (Mitani *et al.,* 2003). Mitani *et al.,* (2003) showed that unless a very high rate of cyazofamid was used in their experiments, they did not get 100 % inhibition of clubbing. Clubbing can form on the roots of brassicas even if only one resting spore infects the roots (Jones and Ingram, 1982). Also, a reduction of primary root hair infection does not give rise to a directionally proportional decrease in clubbing (Naiki and Dixon, 1987). Therefore, for Ranman to be fully effective, the active ingredient would have to come into contact with all spores to prevent them from germinating to be guaranteed complete disease control. This would mean that high rates of Ranman or multiple applications to combat recurrent phases of resting spore germination and subsequent root hair invasion could help improve the control gained when using this treatment.

The results of these experiments would also suggest that even if the spores are capable of germinating when exposed to this treatment, the zoospores are impaired in some way because they cannot complete the secondary stage (cortical stage) of the life-cycle as there were no clubbing symptoms on the tap root. Cortical cells may be protected by Ranman as cyazofamid has low water solubility and thus may not have systemic translocation activity from taproot and root hairs to untreated new hairs (Mitani *et al.,* 2003). Therefore, although new root hairs could be infected by spores, the taproot would continue to be protected. Cyazofamid also has a high degree of soil adsorption and does not leach into deeper soil layers (Mitani *et al.,* 2003) and this may provide the tap root area with a high level of protection. The observation that the secondary stage of the *P.* brassicae life cycle is impared by Ranman also infers that the production of new resting spores would be reduced. Ranman could therefore theoretically reduce the number of resting spores in the soil and prevent the build up of inoculum in field soil. Mitani *et al.,* (2003) also showed that inhibition of root hair infection increased as the resting spore incubation period with cyazofamid increased (Mitani *et al.,* 2003). In these glasshouse experiments, the incubation period was one day before transplanting. If this time period had been increased, the level of control achieved may have been improved.

Ranman was not tested in the 2006 experiment because this fungicide was unlikely to get approval to be used in brassica crops for clubroot control because the manufacturer had no plans to support an application to the Pesticides Safety Directorate for approval for its use against clubroot.

Shirlan

Shirlan was consistently one of the most effective treatments at significantly reducing disease in the glasshouse trials. Shirlan contains the active ingredient fluazinam which also acts by blocking the respiration of the resting spores. Shirlan has been shown to be effective at controlling clubroot in Australia in a field environment and has been recognised to affect *P. brassicae* since the 1980's (Donald *et al.,* 2006). As was the case with Ranman, Shirlan never completely controlled disease (except from in the cultivar Marathon in the 2004 experiment) suggesting that low levels of spores could overcome the effect of this fungitoxic compound. Like Ranman however, Shirlan could also be a useful control treatment in the field because plants exposed to this treatment did not develop disease in their main root system which is where new resting spores are formed.

There was no extra benefit to disease control when calcium oxide or LimeX was used alongside Shirlan. The activity of Shirlan was reduced when it was added alongside calcium carbonate and *P. chlororaphis* together. It has been shown that soil with high sand content, high pH and calcium and high biological activity can reduce the effectiveness of fungicides (Matthiesse and Warton, 2006) and this may be a reason why adding Shirlan along with both a biocontrol agent and a lime treatment to the soil in an attempt to control clubroot was not effective.

A possible explanation as to why Shirlan did not have any noticeable effect on disease control level when it was added to the soil along with limes such as calcium oxide and LimeX could therefore be because the effectiveness of these limes on clubroot control were counteracting any detrimental effect that they were having on Shirlan. Evidence to support this is that these limes completely controlled clubroot when used individually.

Adding Shirlan to the soil on its own or with limes or biological control agents also offered no extra benefit to plant fresh weight. This would suggest that it would be uneconomical to add limes and Shirlan together in a field environment. However, combinations of Shirlan and limes have been used effectively in Australia to control clubroot in fields that contain high spore loads (Donald *et al.,* 2006).

As mentioned, Shirlan was not effective at increasing plant fresh weight. This has also been demonstrated by Cheah *et al.,* (1998) who found that fluazinam, while decreasing disease levels, did not increase head weights of brassicas. For a clubroot control treatment to be of benefit in the field, it would have to provide a benefit to yield otherwise it would be uneconomical to add the treatment. A possible use for Shirlan as a control measure would therefore be to add it to the soil to reduce spore load, perhaps below a certain level that would then support the use of another control measure such as lime in subsequent years. Shirlan has more potential to be launched as an approved control treatment in the UK than Ranman and may be an effective novel control treatment against clubroot commercially.

Amistar

Amistar was only tested for its effectiveness in the 2006 trial and was added to the list of treatments because it is registered for use in brassica crops and is effective against many diseases. It is a broad-spectrum fungicide containing the active ingredient azoxystrobulin which has activity against Oomycota, Ascomycota and Basidiomycota (Sudisha *et al.,* 2005). It acts by inhibiting mitochondrial respiration by binding to the ubihydroquinone oxidation centre of the mitochondrial complex bc_1 (Sudisha *et al.,* 2005). This treatment increased the level of disease on the roots compared to the control plants. Amistar is therefore not effective against the protozoan *P. brassicae* despite the fact that it is acting on the same mitochondrial respiration complex as Ranman which is effective at controlling disease. This would suggest that the ubihydroquinone oxidation centre of complex bc_1 is different in *P. brassicae* compared to Oomycota, Ascomycota and Basidiomycota. Ranman was acting by inhibiting electron transport in the same

complex and could suggest that although there are similarities in the structure of the cytochrome bc1 complex of Oomycete fungi and *P. brassicae,* there may also be slight differences in the way it operates.

Amistar slightly reduced the pH of the soil and so the lower pH may have been providing more optimum conditions for clubroot to develop. Another possible explanation for the increase in disease observed when Amistar was used was that Amistar may have been killing other microorganisms that were present in the soil that could have been natural antagonists of *P. brassicae* and therefore *P. brassicae* would have been able to infect the host plant unchallenged. The likeliness of the presence of natural antagonists of *P. brassicae* in commercial potting compost would be thought of as being low.

Amistar also did not have a positive effect on plant fresh weight and this could be because the level of disease on the roots treated with this treatment was high and the galls were acting as a metabolic sink. Amistar may also have been killing off organisms that helped promote plant growth.

Amistar added in combination with calcium carbonate was better at controlling disease than when the fungicide was added on its own. The most likely cause of this effect lies in the calcium carbonate component which on its own gave better control than when it was added with Amistar. The results also showed that Amistar reduced the effectiveness of calcium carbonate at controlling disease. Perhaps this could suggest that calcium and fungi may act in synergy to control disease in a way that is as yet unknown. Adding *P. chlororaphis* along with Amistar increased the level of disease on the roots compared to when Amistar was used on its own and may show that there is an interaction between bacteria and fungi that occurs naturally to reduce the severity of clubroot.

Conclusions on the future use of fungicides to control clubroot in a field environment

There is a resistance risk with fungicides that act in a site-specific manner such as Ranman, Shirlan and Amistar. Some spores may be able to build up a resistance to these fungicides due to natural selection and therefore, these fungicides might not be an effective way of developing a long lasting durable clubroot control measure. Applying fungicides in combination or rotations may be able to overcome the resistance risk. It has also been shown that the balance of minerals in the soil can affect fungicide uptake (Zadrac *et al.,* 2006). Thus systemic fungicides may vary in their effectiveness in different soil types. The experiments have shown that soil microflora, pH and nutrient content can influence the effectiveness of fungicides against clubroot and so if these treatments are to be used in field conditions, these factors would have to be taken into account.

Limes

Limes are a conventional treatment used to control clubroot and have been used for this purpose for over a hundred years (Dobson *et al.,* 1983). However, limes do not always provide consistent control. There are many different lime products that could be used in the field to control clubroot and the glasshouse experiments aimed to discover the optimum lime treatment that could be used to give the most consistent clubroot control in the field.

Calcium carbonate

Calcium carbonate was consistently one of the most effective treatments at controlling clubroot disease but it never controlled it completely when it was added to the soil on its own. The controlling action of calcium carbonate is attributed to the rise in pH and calcium this treatment provides to the soil. High pH and calcium are known to have a controlling effect and appear to work synergistically to control clubroot (Webster and Dixon, 1991). A higher rate of calcium carbonate was used in the 2005 and 2006 experiment than in the 2004 experiment to determine if this higher level could provide greater levels of control, but this strategy did not effect a greater level of control. This

inferred that there may be an optimum level of calcium carbonate that is needed for clubroot control and adding more than this level will give no further benefit in reducing disease. This was also observed by Murakami *et al.,* (2002).

The calcium carbonate treatment was highly reactive in the soil because it was in the form of a fine powder with a large surface are. It was added to the soil at transplanting in the 2004 experiment and two weeks in advance of transplanting in 2005 and 2006. There was no improvement in disease control when this treatment was added two weeks in advance compared to at transplanting. This would confirm the work of Campbell and Myers (1985) and Larson and Walker (1934) who demonstrated that adding calcium carbonate to the soil one to three days in advance of transplanting gave just as good control as if it was added four weeks in advance of transplanting.

A possible explanation for the calcium carbonate treatment not being totally inhibitive towards *P. brassicae* could be due to the fact that this treatment did not increase pH to a high enough level to get complete control. The final soil pH caused by this treatment was pH 7.1 and pH 7.2 is generally regarded as the optimal level of pH that inhibits clubroot development (Campbell *et al.,* 1985). Calcium oxide and LimeX were more effective than calcium carbonate at increasing soil pH and both of these limes were more effective than calcium carbonate at controlling disease.

The controlling effect of calcium carbonate was reduced when it was added in combination with Biohumate, Quillaja, *P. chlororaphis* and Amistar. These treatments may have been altering pH level which may in turn have affected the control achieved when using calcium carbonate on its own. Another explanation could be that the effectiveness of calcium carbonate as a control treatment for *P. brassicae* is involved in some way with the soil microflora. This is because *P. chlororaphis* is a bacterial strain that produces fungitoxic compounds; Quillaja promotes certain bacteria in the soil and would kill others by lysing membranes; Biohumate also promotes soil microflora, and

Amistar is a broad-spectrum fungicide. This would again suggest that microorganisms and calcium may be interacting in some way when it comes to control of *P. brassicae*.

Calcium carbonate was more effective at controlling disease when added to the soil along with Borax than when it was added on its own. This could be because calcium and boron act together in strengthening cell wall bonds in the middle lamellae (Hepler, 2005) and could have strengthened root cell walls thereby preventing the physical penetration of the zoospores into the root cells.

LimeX

LimeX (also known as sugarbeet waste lime) was tested in the 2005 and 2006 experiments as an alternative to quarried lime and gave complete control against clubroot in both years. This was the only treatment to give complete control against the disease in different experiments. A lower rate was used in 2006 than in 2005 to make the rate tested more comparable to a rate that would be used in a field environment. Perhaps an even lower rate of LimeX would have been able to give complete control but this would require further testing.

LimeX used in combination with fungicides and *P. chlororaphis* also gave complete control. This was different to other lime compounds whose controlling effect generally declined when they were added to the soil along with other treatments, especially *P. chlororaphis*. This observation could provide an important consideration when deciding on a form of lime to use in a field environment. This is because a lime treatment that has a controlling effect that is unaffected by soil microflora or other soil inputs would be beneficial for use in the field. The combination of LimeX and other treatments also gave a slight but non significant increase in plant fresh weight and therefore adding LimeX and other treatments in combination could be a potential strategy for both controlling clubroot disease and increasing plant fresh weight in the field.

The complete controlling effect of LimeX could be because unlike other limes, it contains a high proportion of other plant nutrients such as silicates, phosphorus, magnesium and sulphur. Nutrients such as magnesium, have been shown to have a controlling influence against clubroot (Walker and Hooker, 1945). The presence of increased nutrients in the soil due to this treatment may also improve plant health making the plants more able to respond against infection. Different bacteria and fungi also have different nutritional requirements (Buscot and Varma, 2005) and so these nutrients could have been promoting antagonistic organisms in the soil that may provide protection against *P. brassicae*. Therefore, the rapid increase in soil pH and calcium content that the LimeX supplies, along with the added benefit of various nutrients that interact both with the soil microflora and plant nutrition could be the reason for the increased disease control from LimeX compared to quarried calcium carbonate in the glasshouse.

Calcium oxide

The fineness of lime used in clubroot control has been demonstrated to influence the level of control that can be achieved by the lime (Dobson *et al.,* 1983). Therefore, three different finenesses of calcium oxide treatments were tested for their effectiveness at controlling clubroot in these experiments in an attempt to discover which fineness of lime would give the largest reduction in disease levels. The calcium oxide treatments tested were a very fine powdered calcium oxide, calcium oxide in the form of pebbles and thin flakes of calcium oxide.

The gravel form of calcium oxide used in 2004 was not effective at reducing disease and slightly increased the disease compared to the controls. The flaked lime (QuickCal) used in 2006 also did not control disease. The powdered calcium oxide used in 2005 and 2006 completely controlled disease. This infers that the fineness of the lime used has a major effect on clubroot control. The calcium oxide treatments raised the pH of the soil to high levels. QuickCal added in combination with Perlka raised the pH to 8.9 which was the highest pH reached out of any treatment analysed. The high pH that this

treatment provided in the soil may have provided the controlling effect against clubroot spores. High pH is said to reduce resting spore germination (Colhoun, 1953) and a pH of above 7.2 cause primary thalli to abort before they release secondary zoospores hence reducing clubbing (Myers and Campbell, 1985). Calcium oxide also releases heat energy that may lyse resting spores and denature spore membranes (Heinonen-Tanski *et al.,* 2006). Due to the fact that calcium oxide raises the pH of the soil to a high level and releases heat energy, it could be hypothesised that this treatment would negatively affect root growth. However, this treatment did not significantly reduce plant fresh weight in the experiments and Campbell *et al.,* (1985) also noted that calcium oxide had no effect on plant fresh weight. High pH levels can also affect the solubility of different metals (Buscot and Varma, 2005) but in these experiments this fact did not appear to affect the plants in an adverse manner. Calcium oxide also raises soil calcium levels and calcium has a controlling effect against clubroot (Myers and Campbell, 1985).

The fine powdered form of calcium oxide had a larger surface area than the other forms of calcium oxide and therefore would react more quickly in the soil to raise pH and calcium levels. This reinforces the work carried out by previous researchers who have noted that the finer the lime the better the control (Dobson et al., 1983). Fine limes could come into contact with more spores or could be creating a more even spread of high pH conditions throughout the soil which would make it more difficult for the spores to germinate. The roots would also be in greater contact with high pH and calcium and this may have been having an effect on the level of control that was achieved with this treatment.

Summary of lime treatments

LimeX70 and powdered calcium oxide were the most effective of the lime treatments at controlling disease. The results inferred that the fineness of the lime was critical in achieving good control. The powdered calcium oxide lime was also more effective at controlling disease than the powdered calcium carbonate form and this could have been because the calcium oxide treatment raised the pH of the soil higher than calcium

carbonate, providing greater control by making spore germination conditions less favourable. Calcium oxide also had the added effect of providing heat that could kill the spores which calcium carbonate does not. The use of fine powdered calcium oxide in a field environment may not be acceptable to growers (Richard Haacker pers. comm.) and therefore LimeX70 could provide the best option for a lime treatment used to control clubroot in the field. LimeX70 completely inhibited disease in all of the experiments it was tested in. Its effectiveness at controlling disease was also unaffected by other treatments such as fungicides and *P. chlororaphis* which was not the case with the other limes tested in combination. It could therefore be hypothesised that this lime would be less affected by varying soil properties than the other limes and may possibly give more consistent disease control than the quarried limes.

Nutrients

The balance of nutrients present in soil has been shown to have an effect on clubroot development (Donald *et al.,* 2006a). Therefore, enhancing the nutrient level in the soil using selected fertilisers and individual elements was tested in the glasshouse trials in an attempt to reduce clubroot severity and increase plant growth.

Seaweed products

Two types of seaweed products were tested for their control against clubroot. BioMagic was a fertiliser made from seaweed that had added cytokinins to promote plant growth. This treatment was tested in 2005. It reduced disease to some extent but not significantly and did not have a significant effect on plant fresh weight. Bod Ayre seaweed was a granular product made from dried bladder weed and it had the same effect as the BioMagic treatment on disease and plant fresh weight in the 2006 experiment. These treatments did not raise soil pH and therefore the conditions for clubroot to develop were optimum despite these treatments being added to the soil. Seaweed adds nutrients to the soil and therefore these treatments may have improved plant nutrition so that they were more able to produce defence responses against pathogen invasion which slightly but not significantly reduced disease severity.

In soils with increased nutrients, formation of associations between dark septate endophytic fungi and their host plant was suppressed (Hashiba and Narisawa, 2005). This could infer that associations and infection of host plants by various microorganisms can be affected by the nutrient balance in the soil. Cytokinins are involved in clubroot development (Devos *et al.,* 2005), therefore, adding a seaweed that contained added levels of cytokinins as in the case of the BioMagic treatment may have been counter-productive to disease control because it could have been promoting new plant root growth for the spores to enter.

Biohumate

Biohumate was added to the soil in the initial 2004 experiments because it was a biostimulant. It combined the properties of humic acid with a plant glycoside which work together to improve the physical and chemical nature of the soil and at the same time increase the activity of the beneficial microorganisms around the root zone. It was marketed as being able to increase cation exchange capacity, increase nutrient absorption, reduce nutrient leaching and increase stress tolerance and plant vigour (www.biotechnica.co.uk). It was added to the soil along with other treatments such as potassium tetraborate and calcium carbonate because it was thought that the Biohumate would make these nutrients more available to the plants and as calcium and boron play a part in clubroot control, it was expected that this treatment would be of benefit in controlling disease.

The results of the 2004 experiment demonstrated that Biohumate was phytotoxic and significantly reduced plant fresh weight. This may have been because Biohumate is a very alkaline substance and a large pH increase could have damaged the plant roots causing the plants to die. In pots, treatments cannot drain down the soil profile like they do in the field. This would suggest that using the field rate of this substance in pots could have been too high and concentrated around the roots and was therefore toxic.

Biohumate was less phytotoxic when it was added to the soil along with potassium tetraborate than when it was applied on its own, but this combination was the least effective at controlling clubroot. When Biohumate was added to the soil along with calcium carbonate it was better at providing control and was not phytotoxic. However, calcium carbonate was more effective at controlling disease when it was added to the soil on its own which suggested that the Biohumate treatment was reducing the positive controlling effect of calcium carbonate.

It has been shown that soils with high humic content promote clubroot disease (Murakami *et al.,* 2004). Biohumate is based on humic acid derivatives and therefore this may be a reason as to why it was not effective in controlling clubroot. Humic acids increase cell permeability (Atiyeh *et al.,* 2002) and this weakening of the cells could have allowed the zoospores of *P. brassicae* to enter the roots more easily. Humic acids also promote plant root growth and so they could have been promoting clubroot by providing more roots for the pathogen to enter (Atiyeh *et al.,* 2002). It also promotes plant shoot growth and this could have provided more metabolites for *P. brassicae* development, hence causing large amounts of clubbing on the roots. Humic materials exhibit auxin-, gibberellin-, and cytokinin-like activities (Atiyeh *et al.,* 2002). Auxin and cytokinin also play a role in clubroot development (Devos *et al.,* 2005) so it could be suggested that humic substances are promoting clubroot by increasing plant growth in the same way that auxin and cytokinin does. Soils with a high organic content also promote clubroot disease and organic soils have high levels of humic substances. This again could support the theory that humic acids are promoting clubroot disease. Humic acids also promote soil microorganisms that could also be interacting with *P. brassicae* or the plants in an as yet unknown way to increase disease.

If a level of Biohumate could be found that was not phytotoxic then this treatment could be a good way of testing the humic acid enhancement of clubroot theory. If Biohumate increased the level of disease in the roots then it could be shown that humic acids are

important at promoting clubroot disease and control methods could be developed that could reduce the clubroot promoting effects of humic acids.

Boron

Boron has been shown to have a controlling effect on clubroot and its addition to field soil is currently suggested as a control measure (Webster and Dixon, 1988). Different forms of boron were tested in this trial to determine if there was a form of boron that was more effective at reducing disease than others. Boron was also added to the soil along with other combinations of nutrients to determine if combinations of nutrients could provide greater levels of disease reduction than if they were added on their own.

The boron used in the 2004 experiment was a powdered form of potassium tetraborate. On its own this treatment did not have a significant effect at reducing disease despite the fact that there was evidence that boron has a controlling effect on clubroot (which is discussed in more detail in Chapter 1). Potassium has been shown to promote clubroot disease (Samuel and Garrett, 1944). Potassium antagonises the uptake of calcium (Gibson *et al.,* 2001) and as calcium has a proven controlling effect on clubroot (Myers and Campbell, 1985), the reason why the potassium tetraborate treatment did not control disease was possibly due to the potassium component of this treatment.

Potassium tetraborate was better at controlling disease when it was added with calcium carbonate, but calcium carbonate was more effective at controlling disease on its own. A possible explanation for this was that the potassium component of the treatment was promoting disease whereas the calcium carbonate was preventing it and therefore the potassium tetraborate was counteracting the controlling effect of the calcium.

The potassium tetraborate form of boron was not effective in the 2004 experiments and therefore Borax (sodium tetraborate) was the form of boron used in future experiments. This form of boron was much more effective at controlling disease than potassium tetraborate. Borax was not as effective as calcium carbonate at controlling disease but

when Borax and calcium carbonate were added to the soil together, this combination of treatments was more effective at controlling disease than either treatment on its own. This may be because calcium and boron act in synergy to strengthen plant cell walls (Webster and Dixon, 1988) and this may have prevented the physical entry of the zoospores into the root hair. Sodium has also been observed to have a controlling effect on clubroot (Samuel and Garrett, 1944).

In the 2006 experiment examining the effect of many combinations of treatments on clubroot, Borax was slightly less effective at controlling clubroot when *P. chlororaphis* was added along with it. The reasons for this were unclear but *P. chlororaphis* may have been in competition with the plants for boron or may have affected the mobility or uptake of boron. When Borax, calcium carbonate and *P. chlororaphis* were added together, disease was completely controlled. The wall strengthening effect that the boron and calcium components had, plus the antifungal compound that *P. chlororaphis* produced, could all have been working additively to provide control. This may have been because any spores that were not killed by the antifungal compound would then face a strengthened cell wall. Therefore, two different stages of the life cycle could have been affected by this combination of treatments. The combination of Borax, calcium carbonate and *P. chlororaphis* also slightly reduced plant fresh weight however and the reasons for this were unexplained. The combination of Borax and calcium carbonate had a positive effect on plant growth and would suggest that the *P. chlororaphis* treatment was therefore interacting with the calcium and boron in a way that was detrimental to plant health.

The fact that one form of boron controlled disease while the other did not demonstrated the importance of selecting the correct form of nutrients used in clubroot control strategies. These results again infer that the balance of nutrients in the soil is important in achieving good clubroot control.

Calcium nitrate

Calcium nitrate was tested in the 2006 experiment because it had been previously demonstrated that the calcium ion alone without an increase in soil pH could control clubroot (Myers and Campbell, 1985). Calcium nitrate may also have been effective against disease because this treatment strengthens cell membranes (Borowski *et al.,* 2006) and therefore could have strengthened the plant cell wall against infection from the zoospores.

Calcium nitrate did not increase soil pH or the calcium levels in the soil and had no effect on plant disease. This would suggest that both an increase in calcium and pH is required for clubroot control. Calcium nitrate was one of the few treatments tested in the glasshouse that significantly increased the fresh weight of the plants and could therefore overcome the detrimental effects that the clubbing caused by *P. brassicae* had on the plants. This fresh weight increase was most likely due to the nitrogen that this treatment added to the soil. Nitrogen is also said to have a controlling effect on clubroot (Colhoun, 1961) but this was not seen with this treatment in these glasshouse experiments and therefore the form of nitrogen and the rate that is added to the soil may be the key to control.

Gypsum (calcium sulphate)

Gypsum (calcium sulphate) was tested in the 2006 experiment because it was suspected that the increase in calcium it would provide to the soil would have a controlling effect. Fletcher *et al.,* (1982) reported that calcium sulphate did not increase soil pH but in spite of this, the level of disease incidence was significantly less in clubroot infested soil amended with calcium sulphate than in untreated soil. Webster and Dixon (1991) suggested that calcium sulphate could affect *P. brassicae* during both the primary and secondary phase of development because calcium sulphate applied during either primary or secondary colonization limited club development. Fletcher *et al.,* (1982) also mentioned that calcium sulphate increased crop yield. In the 2006 experiment, gypsum slightly decreased the pH of the soil but slightly increased the calcium level. This

treatment had no effect on disease or plant fresh weight. This contradicted the work of Fletcher *et al.,* (1982) and Webster and Dixon (1991) and showed that clubroot control could not be achieved under these glasshouse conditions using gypsum.

Copper carbonate

Copper carbonate was tested in the glasshouse as a possible clubroot control measure because copper salt had been shown previously to be effective at controlling clubroot (Huber, 1990). It was hypothesised that the broad-spectrum biocidal effect of copper may inhibit *P. brassicae* development by causing oxidative stress towards the organism and altering its uptake of metals. The copper carbonate treatment had no significant effect on disease level, plant fresh weight or soil pH in the 2006 experiment. In some cases it was phytotoxic and it made the tap root look woody and abnormal. The general toxicity of a high level of copper may therefore have been adversely affecting plant metabolism and development.

Perlka

Calcium cyanamide has been used for over 100 years as a slow release N-fertiliser, herbicide, pesticide and fungicide (Williamson and Dyce, 1989) and is known to have a controlling effect against clubroot (Williamson and Dyce, 1989; Donald *et al.,* 2004). Perlka has often been shown to be phytotoxic if applied too close to planting and at too high a level (Williamson and Dyce, 1989) so the aim of testing this treatment in the glasshouse was to optimise rates and timings of addition of this treatment so that it would give the greatest levels of clubroot control without being phytotoxic. This knowledge could then be used to optimise the level of control achieved by Perlka in a field environment.

In the initial screening of treatments in the 2004 experiment, Perlka (a granular form of calcium cyanamide) significantly controlled disease in the Monaco cultivar of calabrese and reduced the level of disease in the Marathon cultivar but not significantly. This would add more evidence to the observations made by Williamson and Dyce, (1989)

that calcium cyanamide could be toxic to some plant cultivars and not others. The reason for this was unclear but demonstrates that some plant cultivars may be more tolerant to the toxic compounds that Perlka produces than others.

In the 2005 experiment which was used to optimise rates and timings of addition of treatments, a higher rate of Perlka was tested to determine if this higher rate would provide a higher level of disease control. This higher level was seen to be phytotoxic. The reason for this was most likely due to the fact that the plants were transplanted into the soil one day after this treatment was added, and therefore the high level of nitrogen in the soil that this treatment provided was likely to be toxic to the plants. Williamson and Dyce, (1989) also showed phytotoxicity of Perlka due to nitrogen toxicity when high levels of the compound was used and added to the soil close to transplanting. Perlka degrades to hydrogen cyanamide, urea, dicyandiamide and hydrated lime when it contacts water (Donald *et al.,* 2004). When Perlka was added to the pots at a high rate, these substances would have been very concentrated around the roots as they would not be able to drain down the soil profile like they would be able to in the field. Any of these substances could have been phytotoxic at high levels.

The 2006 experiment was again used to refine rates and timings of addition of treatments and also to examine the effect of adding treatments to the soil in combination for disease control. Perlka was more effective at controlling disease when it was added five days in advance of transplanting and at a much lower rate in this experiment compared with the 2005 experiment. These glasshouse experiments concur with the work of previous researchers who demonstrated that the timing of addition of Perlka is important in its effectiveness as a clubroot control measure (Williamson and Dyce, 1989; Donald *et al.,* 2004).

Tremblay *et al.,* (2005) observed that Perlka was effective at controlling disease when it was added to the soil one week in advance of transplanting but was less effective when it was added to the soil 14 days in advance of transplanting. Perlka has been shown to be

toxic to spores because of the compound hydrogen cyanamide that is produced when Perlka comes into contact with water (Williamson and Dyce, 1989). Hydrogen cyanamide is also produced by many species of plants in response to tissue damage (Osbourn, 1996) and certain fungi can detoxify hydrogen cyanamide (Chini *et al.,* 2005). Perhaps when this treatment is added to the soil far in advance of transplanting, the level of the toxic substance is reduced in the soil due to its detoxification and therefore *P. brassicae* spores could recover over time and infect the roots of host plants. Naiki and Dixon (1987) showed that incubating spores with Perlka for one day reduced their viability, however, and Donald *et al.,* (2004) showed that hydrogen cyanamide increased in proportion to the interval between incorporation and planting.

Plants treated with Perlka in the 2006 experiment were significantly higher in fresh weight compared to control plants and this may have been due to the nitrogen that this treatment added to the soil. This treatment did not increase soil pH or calcium levels despite the fact that calcium hydroxide was released from this compound on contact with water, and this again adds evidence to previous research that Perlka controls clubroot due to the fungitoxic compound that it produces and not due to the calcium that it contains or its effect on soil pH.

When Perlka was added to the soil along with the flaked calcium oxide treatment (QuickCal), disease was completely inhibited. The Perlka and QuickCal treatment combination gave the highest soil pH out of all of the treatments tested and this high pH may be one of the main reasons why this treatment combination was so effective at controlling disease. These two treatments may have been acting on clubroot at different stages in its development (Perlka on the spores and QuickCal on the zoospores or root hair stages) and therefore provided an additive effect on disease control. These results demonstrated that if Perlka was added to the soil in combination with a lime treatment that raised soil pH to a high level, not only was disease completely inhibited, but a significant increase in plant fresh weight was achieved. This combination of treatments

would therefore be hypothesised to be an effective method to increase yield and prevent clubroot in the field.

Summary of the effect of nutrients

Although promoting plant health by adding a balanced level of nutrients to the soil, or adding nutrients to the soil that have previously been shown to have a clubroot controlling effect would be hypothesised to be beneficial in disease control, many of the treatments that added nutrients to the soil (including seaweed-based fertilisers and potassium tetraborate) had no effect on disease. It would appear to be better to add nutritional treatments like Perlka that also have fungitoxic attributes to achieve a greater level of control.

Adding a treatment to the soil that would be expected to make certain nutrients more available to the plants (Biohumate) was ineffective at controlling clubroot and the humic acid component of this treatment may have even been promoting disease.

The results also suggested that increasing calcium level alone was not effective at reducing clubroot and that an increase in both pH and calcium level in the soil may be needed for clubroot control. This would agree with (Webster and Dixon, 1991) but would contradict (Myers and Campbell, 1985) who reported that the calcium ion alone could control clubroot.

The results also demonstrated that the form of a particular nutritional element could be critical in achieving clubroot control because different forms of boron varied in their effectiveness at reducing disease. These results again infer that the balance and form of nutrients added to the soil is important in achieving good clubroot control.

Biocontrol agents

Bactolife products

The Bactolife products tested in the glasshouse trials were commercially available powders containing mixtures of bacteria, fungi and yeasts that were marketed as promoting good soil health (www.biotechnica.co.uk). These organisms may produce fungitoxic compounds which could have the potential to destroy spores. They may also benefit plant health and would therefore make the plants less susceptible to infection by *P. brassicae*.

Although the Bactolife products appeared to have been successful at controlling disease in the initial 2004 experiments, they also caused the plants to die. Therefore, the reason that there was low levels disease on the roots of plants exposed to these treatments could have been because the roots were not growing properly and clubroot zoospores only infect growing roots, especially roots that are five to six days old (Asano *et al.,* 2000). The reason that these treatments were toxic to the plants may have been because the level of the product added to the soil was too high. There may have been competition between the plants and the microorganisms for resources such as oxygen and nutrients. The microorganisms may also have been producing secondary compounds that overpowered the plants at high levels (Mallik, 2001). The substances used to activate this powdered mixture of microorganisms included sugar and this sugar may also have been phytotoxic. In pots, treatments may not be able to drain down the soil profile like they can in field soil causing the products to become very concentrated around the roots.

Lower rates of the Bactolife products were used in the 2005 experiment to try and reduce any phytotoxic effects of the high initial rate of microorganisms in the soil. The treatments were also added to the soil two weeks in advance of transplanting. By adding the treatments two weeks in advance of transplanting it was hypothesised that the microorganisms would have more time to create antifungal compounds that could adversely affect the clubroot spores before transplanting. It was also hoped that the

microorganisms would become balanced in the soil before the plants were added creating a less stressful environment for the plant roots. In the 2005 experiments the lower rate of Bactolife products did not give good disease control but they were not phytotoxic. This demonstrated that the rate and timing of addition of these treatments could be optimised to give a better effect on plant fresh weight.

Bactolife DP104 was slightly more effective at controlling disease than Bactolife S. Bactolife DP104 differs to Bactolife S in that it contains the same bacterial mix as Bactolife S but also has fungi and yeasts. This result could suggest that certain fungi may hinder disease development.

Bactolife DP104 was tested again in the 2006 experiment and did not significantly reduce disease. The pH of the soil was also slightly lowered when exposed to this treatment and therefore the pH conditions for clubroot development were optimal when treated with this treatment. This may be a reason as to why the Bactolife products were not effective at significantly reducing disease.

Pseudomonas chlororaphis

Pseudomonas chlororaphis is a strain of bacteria that controls root rot caused by *Fusarium* oxy*sporum*. It does this by producing the antifungal compound phenazine-1-carboxamide (Chin-A-Woeng, 2001). The compound phenazine also controls *Alternaria solani* and *Pythium aphanidermatum* by degrading their cell contents and lysing their hyphae (Kavitha *et al.,* 2005). It also completely inhibits the germination of *Rhizoctonia solani* sclerotia and can suppress the hatching of *Meloidogyne incognita* eggs (Kavitha *et al.,* 2005). *Pseudomonas chlororaphis* was therefore tested in the glasshouse to determine if it could have a similar controlling effect against *P. brassicae* resting spores.

A rate of 10^9 CFU/ml of *P. chlororaphis* was effective at significantly reducing clubroot in cultivar Monaco in 2004 but not cultivar Marathon. This may be due to the fact that cultivar Marathon was already stressed by an aphid attack. The reduction in plant health

could have meant that these plants would have been more easily infected and less responsive to the benefit of a clubroot control measure.

In 2005, different rates of *P. chlororaphis* were added to the soil to determine if a different rate would provide a higher level of clubroot control. It was observed that lower levels of *P. chlororaphis* were slightly more effective at controlling disease than higher levels but this was not significant. It could have been hypothesised that higher levels of *P. chlororaphis* would have produced higher levels of phenazine-1-carboxamide which would therefore have been expected to come into greater contact with *P. brassicae* spores hence promoting a greater level of disease control. This was not the case in these experiments. Therefore, a possible explanation for the results is that there may be an optimum level of phenazine-1-carboxamide that has a controlling effect against clubroot but if more than this optimum level is produced by *P. chlororaphis* there may be no more additive effect.

The fresh weight results of the 2005 experiment demonstrated that as higher levels of *P. chlororaphis* were added to the soil, there was a detrimental effect on plant weight. Although this was not significant, a rate of 10^6 CFU/ml was used in the 2006 experiment because this rate gave the best fresh weight results of the rates tested.

In the 2006 experiment, *P. chlororaphis* was added to the soil in combination with other treatments and generally reduced the effectiveness of these other treatments at controlling disease. It reduced the effectiveness of Amistar, Borax, calcium carbonate, calcium oxide and Quillaja. It slightly increased the effectiveness of shell sand, Shirlan, spent mushroom compost and the combination of Borax + calcium carbonate. This demonstrated that certain microorganisms may be able to interact with clubroot control measures in the soil and could make them more or less effective. Therefore, differences in soil microflora may be a reason as to why there are so many contradictory results regarding the level of clubroot control achieved in different soil when the same treatments have been used.

An experiment by Thomas *et al.,* (2000) showed that *P. chlororaphis* 1391 was impaired in root tip colonisation in a sand system and in potting soil and that this reduced its effectiveness at controlling tomato root rot. This could suggest that root colonisation by bacteria may play a crucial role in biocontrol. Therefore, different soil types may create unfavourable conditions for certain microorganisms that hinder clubroot development to attach to roots, therefore affecting the level of disease control achieved in that soil.

Pseudomonas chlororaphis is already approved for use on cereal seeds and if it was determined to have a significant controlling effect against clubroot in the field, it could possibly be used in organic farming systems as an alternative to fungicides.

Pseudomonas fluorescens

Fluorescent Pseudomonads belong to the plant growth promoting rhizobacteria (PGPR) and play a role in plant growth promotion, induced systemic resistance and biological control of pathogens (Ganesham and Manoj-Kumar, 2005). Bianciotto *et al.,* (1996) demonstrated that *P. fluorescens* WCS 365 (as used in the experiments in this study) was one of the most effective colonisers of the spores and mycelium of *Gigaspora margarita*. It produced extracellular material containing cellulose to attach itself to the spores and this substance may have been mediating fungal/bacterial interactions. Fluorescent pseudomonads also produce extra-cellular surface active metabolites (biosurfactants) (Stanghellini and Miller, 1997) which may have an effect at lysing spores such as the spores of *P. brassicae*.

Pseudomonas fluorescens provided some controlling effect against clubroot but this was not a significant level of reduction in disease. The 2005 experiment showed that low levels of *P. fluorescens* were slightly but not significantly better at controlling clubroot disease than high levels. Lower rates of the bacterium also had a more beneficial effect on plant fresh weight. These were the same results that were observed with the *P. chlororaphis* treatment. This may suggest that lower levels of individual bacteria can prevent disease development and increase plant growth more effectively compared with

higher levels. The reasons for this are unknown but a possible explanation could be that high levels of bacteria may have been in competition with the plants for oxygen and nutrients and therefore the plants may not have been able to take up enough nutrients to make defence products and protect itself against invasion from *P. brassicae*. High levels of bacteria may also have been producing high levels of compounds that may have been phytotoxic.

Specific pseudomonads are known to increase in number when *P. brassicae* resting spores are present in the soil (Hjort *et al.,* 2007). The reason for this is unknown but there may be interactions between these organisms and *P. brassicae* that could be exploited for the development of future biocontrol agents against clubroot.

Summary of biocontrol agents as an effective clubroot control treatment

The balance of microorganisms in the rhizosphere is complicated and the effect that the soil microflora has on clubroot development and therefore on disease control is not fully understood. Soil microflora is known to have an effect on clubroot because Castlebury *et al.,* (1994) observed that *P. brassicae* spores that had been disinfected could not germinate. Therefore some organisms may promote clubroot disease by promoting resting spore germination. The level of chitinolytic bacteria and pseudomonads in the soil have also been observed to increase after the addition of *P. brassicae* spores to the soil (Hjort *et al.,* 2007). These types of organism may also be interacting with *P. brassicae* in promoting or hindering disease development. Buckzacki (1983) also showed that there were enhanced root hair infections when root hairs were suspended in non-sterile soil. The factor responsible for this enhanced germination was found to be a protein that was induced by other soil microorganisms and its effects were mimicked by treating spores with proteolytic enzymes. This would suggest that an increase in infection may have been due to the breakdown of proteins localised in the outer wall of the resting spores by certain microorganisms. Until the organisms that have these sorts of effects on clubroot are discovered and their effects are fully understood, trying to find the right balance of organisms to reduce clubroot in a field environment would be

extremely difficult. The interactions between soil microflora and treatments such as lime that are added to the soil for clubroot control are also not well understood. The interaction of soil microflora and conventional treatments such as lime with *P. brassicae* would be a good area of research for future work into the effectiveness of clubroot control measures.

Novel treatments – waste products from industry

Alternative treatments to lime that were readily available and classed as waste products from other industries were tested in the glasshouse to determine if they could be used as a novel and sustainable approach to control clubroot.

Shellfish waste products

Chitin

Chitin is a linear polysaccharide of N-acetylglucosamine. It is the second most abundant natural polymer after cellulose. The main biological function of chitin is support as it forms part of the cell walls of fungi and invertebrates and is also found in the cell wall of *P. brassicae* (Moxham, 1983). Chitin provides a food source for certain fungi and bacteria. Therefore, if a large amount of chitin is present in the soil, the number of chitin degrading species in the soil increase (Adam *et al.,* 2001). It was hypothesised that if a chitin treatment was added to the soil in the glasshouse experiments, these chitinaceous organisms would increase in level, consume the chitin in the soil, and then consume the chitin in the cell walls of clubroot spores, destroy the resting spores and hence reduce disease. Chitin has been used as an organic amendment and fertiliser for many years and may demonstrate potential as a clubroot control treatment in the field if preliminary glasshouse tests determined that it had an effect against disease.

The chitin treatment used in the 2004 experiments consisted of prawn shells that had gone through a lactic-acid bacteria bio-processing treatment (Healy, 2006). This powdered chitin treatment significantly reduced disease in both of the calabrese cultivars

tested and it could be suggested that the chitinolytic organisms in the soil did degrade the spores. This would be unlikely however because the treatment was added to the soil one day before the plants were transplanted into the soil and therefore the chitinolytic bacteria in the soil would probably not have reached high enough levels in the soil to have degraded all of the excess chitin and therefore would have been unlikely to have degraded the chitin in the cell wall of *P. brassicae* resting spores.

Another more likely explanation for the controlling effect of the chitin treatment is that the breakdown of chitin by the chitinolytic organisms produced ammonia which could have a controlling effect on clubroot. Rodriguez-Kabana *et al.,* (1984) showed that nematode cysts became permeable when 1% (w/w) chitin was added to the soil and Khan and Khan (1995) suggested that they were destroyed by the ammonia content of the chitin.

Another possible explanation for the controlling effect of chitin could be that the chitin was eliciting a defence response in the plants that would make them more able to prevent infection by the *P. brassicae* spores. Chitin is known to promote defense responses in plants that are attacked by insects (Salzer *et al.,* 2000). The chitin containing *G. mosseae* also induces chitinase activity in *B. napus* roots (Velerheilig *et al.,* 2000).

Rodriguez-Kabana *et al.,* (1984) showed that the growth of soybean roots decreased with increased levels of chitin and Spiegel *et al.,* (1988) obtained similar results with cabbage plants. If the same reduction in root growth occurred in the calabrese plants, this may have given less opportunity for clubroot spores to enter the roots and cause disease. Haverkort and Trudgill (1995) recommended a maximum of 0.5 % (w/w) chitin before the effects of the released ammonia became detrimental to plant growth. In the 2004 experiment 1% (w/w) was used and this showed no negative effect on plant fresh weight.

Chitinolytic bacteria have been observed to increase in the soil when *P. brassicae* is present (Hjort *et al.,* 2007) but perhaps these bacteria are facilitating an increase in disease rather than preventing it. Evidence for this is that the resting spores of *P.* brassicae have a closed germ pore plugged by the thickening of the middle wall layer (Tanaka *et al.,* 2001). The middle layer of the cell wall of *P. brassicae* contains chitin and the thickening of the middle wall layer is known to be susceptible to chitinolytic enzymes (Tanaka *et al.,* 2001). Therefore, chitinolytic processes may be involved in the formation of the opened germ pore in the resting spores of *P. brassicae.* The zoospore flagella are produced almost immediately after the first part of the cytoplasm comes through the break in the spore case and the partially germinated spore swims actively (Ellison, 1945). This could mean that chitinolytic bacteria could enhance resting spore germination, releasing the zoospores into the soil ready to infect the host plants.

Salzer *et al.,* (2000) demonstrated that crude chitin was better than colloidal chitin for plant disease control and this could have been because proteins present in the crude form were acting as growth factors or nutrients which would improve the plants ability at increasing defence responses. Therefore, the form of chitin used for control may be an important consideration if developing this treatment further for field use.

Shell sand

Lime which controls clubroot is made from sea shells. Lime adds calcium to the soil and increases soil pH. Therefore, the crushed seashell product made from whelk and scallop shells would have been expected to have a controlling effect against clubroot as it would also be expected to add calcium to the soil and raise soil pH.

Shell sand was tested in the 2005 and 2006 experiments and significantly reduced clubroot in both experiments when added to the soil on its own. The shell sand treatment added the highest amount of calcium to the soil of any treatment analysed for calcium content and provided a soil pH of 7.3, yet did not give a complete controlling effect against disease. This would suggest that although this treatment caused high

calcium and pH in the soil, factors other than just pH and calcium are involved in clubroot control.

This product also had a high nutrient content. It contained elements such as aluminium, sulphur, manganese, iron and zinc. The high level of nutrients that this treatment provided to the soil could also have benefited the plants by making them healthier and less susceptible to infection.

When shell sand was added to the soil along with *P. chlororaphis*, disease was completely controlled. This was one of the few occasions where adding *P. chlororaphis* to the soil along with another treatment improved the controlling effect of that treatment. The reason for this was unknown but it would suggest that shell sand acted differently in its control mechanism compared to calcium carbonate and calcium oxide because the controlling effect of calcium carbonate and calcium oxide was reduced when *P. chlororaphis* was added to the soil along with these treatments. Therefore, the nutrients other than calcium that shell sand provided to the soil may have been having a different effect on clubroot development compared to calcium carbonate and calcium oxide.

Shell sand may be acting in a similar way to the LimeX treatment because LimeX also contained greater levels of nutrients than calcium oxide and calcium carbonate and its controlling effect was unaffected by the addition of *P. chlororaphis*. Nutrients could have an effect on the proportion of microorganisms in the soil because certain microorganisms have different nutritional requirements (Buscot and Varma, 2005). These microorganisms may then have an effect on preventing disease development.

Summary of using shellfish waste products to control clubroot

Both of the shellfish waste products that were tested in the glasshouse experiments significantly reduced the level of clubroot in calabrese. The shellfish waste product tested in the initial 2004 experiment was made of prawn shells and contained a high level of chitin. It was hypothesised that this treatment may have been controlling

clubroot due to the ammonia that it would produce when it degraded. The shell sand treatment was made from crushed whelk and scallop shells that contained a high amount of calcium and it was hypothesised that this treatment would be controlling clubroot by acting in a similar way to lime – by adding calcium to the soil and by increasing soil pH. Therefore, although both of these treatments possibly acted on controlling clubroot in different ways, they could possibly both have merit for being used to control clubroot in a field environment. As they were both waste products from industry, these treatments could provide a new sustainable way to control clubroot.

Spent mushroom compost

Spent mushroom compost is made of chopped straw and poultry manure with water and gypsum. There is then a 5 cm layer of limed peat placed on top (Greenmyre Mushrooms Ltd, pers. com.). It is commonly used as a soil fertiliser (Stewart *et al.,* 1997) and it was hypothesised that it could improve soil nutrient status to the benefit of the plant and to the detriment of the pathogen. Also, because it contained lime it may have been able to control clubroot by increasing soil pH and adding calcium to the soil which are two of the main controlling factors in clubroot (Myers and Campbell, 1985). Another controlling action that could be envisaged by the use of SMC was that it may have contained chitinolytic organisms due to the chitin-containing mushrooms that had previously been grown in it. These organisms could potentially multiply in the soil and degrade the chitin in the cell walls of *P. brassicae* resting spores and therefore reduce disease by destroying the spores.

Spent mushroom compost at 30% (w/w) had a significant effect at controlling clubroot whereas SMC at 10 % was not effective. This demonstrated that altering rates of treatments may be necessary to discover the optimum dosage for clubroot control.

Although SMC was thought to contain chitinolytic bacteria, it may not have been the bacteria that were providing the controlling effect on clubroot (see chitin section above for possible reasons for this). SMC also contained lime and other nutrients that were of

benefit to the plants and therefore, it could be suggested that more SMC provided more nutrients to the plant and the healthier and more able the plants became at preventing disease. The lime portion may also have raised soil pH which would have made soil conditions for clubroot development unfavourable. Supporting evidence for this was that the SMC treatment at 30% (w/w) raised the final soil pH to 7.1 and gave a significant increase in soil extractable calcium levels.

SMC at 10% (w/w) had a more positive effect on plant fresh weight than SMC at 30% (w/w). This demonstrated that an increase in treatment rate could improve disease control but could also become toxic to the plants. This is another reason why rates of treatments would have to be optimised for use in a field environment.

The ammonia content of the SMC produced by the breakdown of chitin may have been phytotoxic as was mentioned in the chitin section above. Another possible explanation for the phytotoxicity of high levels of this treatment could have been because it may have increased the microbial activity in the soil to levels that were detrimental to the plants. These organisms may have been in competition with the plants for resources, causing the plant growth to be reduced.

The effectiveness of SMC at controlling clubroot was increased when added to the soil along with *P. chlororaphis*. The reasons for this are unclear but the same result was seen when shell sand and Shirlan were added to the soil along with *P. chlororaphis*. This would suggest that the controlling effect of SMC is acting in a similar way to these treatments.

Softguard

Softguard was a liquid form of chitosan. Chitosan is the enzyme that degrades chitin polymers (Salzer *et al.,* 2000). It was thought that this product would degrade the chitin in the clubroot resting spores therefore destroying the resting spores making the organism unable to complete its life-cycle and hence reducing disease. Chitosan as an

elicitor has also been shown to increase plant resistance to different diseases (Borowski *et al.,* 2006).

Softguard was added to the soil one day before transplanting in the 2005 experiment. It had no significant effect on disease control or plant fresh weight. In the 2006 experiment, Softguard was added to the soil five days in advance of transplanting because it was hypothesised that the chitosan would have more time to act on the spores and degrade their cell walls. The Softguard treatment again did not have a significant effect on clubroot control or on plant fresh weight in this experiment.

A possible explanation as to why Softguard was ineffective at reducing disease may have been because the chitin in *P. brassicae* resting spores was too deep inside the walls for it to be degraded. This was also suggested by (Moxham, 1983) who observed that the chitin in *P. brassicae* cell walls was deep inside and masked by a layer of proteins, possibly to disguise its presence from plants. Alternatively, Softguard may have degraded the germ pore in the resting spores therefore enhancing the germination of the resting spores giving another possible explanation as to why this treatment was not effective at reducing disease. It also had no effect on soil pH and therefore soil conditions may have been optimal for clubroot development despite the spores being exposed to this treatment.

The results of using liquid chitosan would also add evidence to the theory that the chitin product made from prawn shells that was tested in 2004 and significantly reduced disease was not acting by degrading the chitin in the spore cell walls but was acting in a different way such as increasing the level of ammonia in the soil.

Surfactants

Saponins (surfactants) provide disease control by affecting transmembrane proteins in microorganisms. The proteins are aggregated together by the saponins resulting in areas devoid of proteins, resulting in the membrane being permeable (Baumann et al., 2000).

This allows external toxins and organisms to enter spores which can lead to death. Merris *et al.,* (2003) demonstrated that Quillaja (a natural plant saponin) could control nematodes when the cholesterol in their membranes and the saponin bound together. This affected the motility of the nematodes. In the case of clubroot, saponins could provide control by affecting cholesterol in the clubroot spores and affecting the high levels of lipids in the cell wall (Knights, 1970). Stanghellini and Miller (1997) also suggested that surfactants would be likely to act on the zoospore stage of the life cycle of *P. brassicae*, by also affecting their motility.

Three different types of surfactant were used in these glasshouse experiments. The Quillaja and Yucca treatments were natural plant saponins which are produced by the Quillaja and Yucca plants as a biochemical defence mechanism against pathogen attack (Khafagi *et al.,* 2003). Agral was a synthetic non-ionic surfactant which is used as an adjuvant when adding fungicides to the soil to improve water spread throughout the soil by reducing surface tension. All three surfactants significantly reduced disease in the calabrese cultivar Monaco in the initial 2004 experiment but only Yucca had a significant effect on disease in the cultivar Marathon. This may suggest that plant stress and health plays a big part in the effectiveness of surfactants at being able to control clubroot because the cultivar Marathon was badly affected by aphids.

Quillaja had a slight controlling effect in the 2005 experiments which was used to optimise dose rates, but despite the fact that a higher rate was used than in the 2004 experiments, the reduction in disease control was not improved significantly. This would suggest that there may be a level of Quillaja that had a controlling effect on clubroot and adding more than this level may not provide an enhanced level of disease control. The Yucca treatment was slightly more effective at reducing disease severity in the 2005 experiment than the Quillaja treatments and this may suggest that different saponins act differently in their control mechanism against *P. brassicae*.

Agral had previously been shown to have a clubroot controlling effect (Stanghellini and Miller, 1997). However, in this study, the natural surfactants were more effective than the synthetic surfactant Agral at reducing disease. Different levels of pathogen control achieved using different surfactants was also observed in work with nematodes where the Yucca extract was more effective than Quillaja at controlling infection by nematodes in potatoes (Falconer, 2004). This research demonstrates that there would have to be a careful selection of treatments such as surfactants if they were to be used in clubroot control because treatments that would be expected to have the same effect on disease do not appear to have the same controlling effect in practice.

Surfactants could also provide control by improving water spread in the soil and increasing the availability of nutrients to the plants so that they were healthier and were more able to resist pathogen infection (Laha and Luthy, 2004). *Alternaria solani* counters the effect of saponins by lowering the pH at the infection site to levels at which saponins are ineffective (Osbourn, 1996). Some pathogens enzymatically digest saponins so therefore different microflora may affect the effectiveness of saponins (Osbourn, 1996). Research has also shown that adding nutrients with biosurfactants prevents them from being degraded (Stanghellini and Miller, 1997). In these experiments, Quillaja was slightly more effective when added to the soil along with calcium carbonate than when it was added on its own and therefore, the prevention of its degradation because of the high pH and nutrients that the calcium carbonate treatment provided may be the reason for the result.

Summary of use of surfactants

Natural surfactants from plant extracts were more effective at reducing clubroot than the synthetic surfactant Agral. Therefore, careful selection of surfactants would have to be made to achieve optimal clubroot control in the field as different surfactants may act differently in their mechanism to control disease. It would be difficult for *P. brassicae* to build up a resistance to biosurfactants because it would require a major change in the

make-up of its plasma membrane (Stanghellini and Miller, 1997). Therefore, surfactants could potentially be a good way of preventing clubroot disease in the field.

Plant extracts

Peppermint oil

Peppermint oil has fungitoxic properties and had been shown in studies by ADAS to have a clubroot controlling effect (Peter Gladders, pers. com.). When it was used on its own against clubroot in the 2006 experiment, it had no effect on disease control. When it was added to the soil along with calcium carbonate, disease was completely inhibited. This treatment may have increased the effectiveness of the calcium carbonate treatment at controlling disease because calcium carbonate used on its own never completely inhibited disease in any experiment.

Peppermint/menthol is known to block calcium channels (Swandulla *et al.,* 1986). It inactivates calcium channels in a calcium-dependent manner (Swandulla *et al.,* 1986). Therefore, an interaction between the mint and the calcium carbonate may have occurred to prevent clubroot. A high level of calcium can also block calcium channels by affecting the rate of calcium uptake into cells (Hepler, 2005) and it is known that high levels of calcium can prevent clubroot (Myers and Campbell, 1985). The blockage of calcium channels by peppermint along with a high level of calcium in the soil provided by calcium carbonate may have provided the additive effect at controlling clubroot. The blockage of calcium channels could either be affecting root membrane potential or zoospore membrane potential which could be providing the controlling effect. Calcium can also promote germination of clubroot spores (Yano *et al.,* 1991) and therefore if *P. brassicae* used calcium channels to detect calcium levels for germination purposes, blocking them could potentially prevent germination.

Peppermint oil on its own did not raise the pH of the soil to a high level. When peppermint oil was added along with calcium carbonate, the pH of the soil increased to

pH 7.5 which was higher than when calcium carbonate was added to the soil on its own. This would again suggest that there is an interaction between the peppermint oil and calcium carbonate at altering the pH balance of the root membranes and therefore the soil around it, possibly involving calcium channels. This observation may help in determining the mechanisms behind how clubroot development occurs.

Rhubarb leaves/water

Rhubarb has been suggested to have a controlling effect on clubroot by many gardeners (Smith, 1966). Rhubarb leaves contain a high level of calcium oxalate which is a fungitoxic compound (van Kan, 2005) and therefore rhubarb leaves were added to the soil in the glasshouse to determine if rhubarb had a controlling effect on clubroot under glasshouse conditions.

In the 2005 experiment, ripped rhubarb leaves and water which had had rhubarb leaves boiled in it both had a slight but non-significant effect at reducing disease. These treatments may have been preventing some of the spores from germinating and in turn preventing disease due to the presence of calcium oxalate. The calcium component of calcium oxalate is also effective at controlling clubroot. High levels of oxalate also induce plant cells to carry out a hypersensitive reaction response (Cessna *et al.,* 2000), killing cells which in turn could prevent the development of *P. brassicae* in the roots.

It has been observed that bacteria that can degrade oxalic acid can protect against disease from fungal pathogens that use this compound as a virulence factor (Morris and Allen, 2004). *Phytopthora infestans*, *Botrytis cinerea* and the related white rot fungus *Sclerotinium sclerotiorum* produce oxalic acid at the onset of infection to chelate calcium, disrupt the cell wall structure, lower the pH and set the conditions for the concerted action of cell wall degrading enzymes and other virulence factors (Cessna *et al.,* 2000). It may be possible that *P. brassicae* produces oxalate to break down the cell wall although there is no evidence for this. If *P. brassicae* did use oxalic acid as a virulence factor, adding the rhubarb leaves to the soil with high levels of oxalate

degrading bacteria could theoretically destroy the virulence factor and prevent disease from occurring.

Calcium oxalate also blocks the adsorption of calcium (Franceschi and Nakata, 2005) and calcium-mediated plant responses can also be blocked by oxalate (Franceschi and Nakata, 2005). These factors may be involved in the observation that many vegetable growers have observed about rhubarb being able to reduce clubroot.

In the 2005 experiment, the rhubarb leaves may not have been mixed into the soil thoroughly enough for the calcium oxalate to come into contact with the spores or roots which may be a reason as to why a high level of clubroot control was not achieved.

Summary of effect of plant extracts on disease

The use of natural plant extracts that had previously been observed to have a controlling effect on clubroot such as rhubarb and peppermint oil were not effective at reducing disease. However, it was noted that both of these treatments are known to have an effect on calcium channels. This observation would again support the theory that the uptake of calcium by either the plant or the pathogen plays a major role in the development of clubroot and therefore the control of clubroot.

SIPeco

SIPeco was a disinfectant based on hydrogen peroxide. This treatment was not effective at controlling clubroot showing that it was not effective at killing clubroot spores but may have killed many other microorganisms in the soil.

Difference between control achieved in Marathon and Monaco cultivars of calabrese

In the initial 2004 experiments, two different cultivars of calabrese were used. Marathon is a cultivar that is widely used commercially in England and Monaco is used

commercially in Scotland. The cultivar Marathon was much more susceptible to an aphid attack that occurred in the glasshouse at the time of the experiments. Fewer treatments tested were effective at controlling disease on the Marathon plants than the Monaco plants. The stressed Marathon plants may therefore have in general been more open to infection by clubroot and so the spores may have overcome any beneficial effect provided by the treatments.

The difference in the responsiveness of the cultivars of the calabrese to the controlling effects of various treatments may also have been because of the different genotypes of these hosts. Different thresholds of spores might have been needed for infection in both cultivars resulting from specific or non-specific interactions with the pathogen (Williamson and Dyce, 1989). The cultivar Monaco may genetically be more resistant to clubroot disease or more responsive to treatments because of its genotype. Cultivars with a strong tap root also have better resistance (Wallenhammer *et al.,* 2000) showing that there may be a difference in the effectiveness of treatments to control disease in different plant cultivars. This may be a reason as to why there is contradictory research about the effectiveness of clubroot control measures in different plants.

Throughout these glasshouse experiments, a reduction in disease level did not equate to an increase in fresh weight. This may be because the conditions were optimum for plant growth in the glasshouse and the plants could overcome the deleterious effects of the disease by taking up adequate water and nutrients effectively.

It was also often noted that plants with large galls also had a higher fresh weight and this may have indicated that the disease was having an effect on plant growth by perhaps inducing plant growth hormones and enhancing its metabolism. This would confirm research carried out by Devos *et al.,* (2005) who demonstrated that plants had enhanced growth in the early stages of infection by *P. brassicae*.

Glasshouse experiments can show more dramatic results and better control can be gained than in the field experiments. This is because more factors can be controlled in the glasshouse than in the field and the levels of treatments are more concentrated in pots than in field soil, hence disease reduction is likely to be greater in the glasshouse.

Less disease was seen on the 2006 experiment roots compared to previous experiments and this may have been because the spores that were used to inoculate the pots had been passed through many rounds of infection in a Chinese cabbage host to get enough inoculum for such a large experiment. Passing the spores through such a susceptible host may have caused the spores to become more adapted to infecting Chinese cabbage and were therefore not as infective on the calabrese host used in the experiment. Crute *et al.,* (1983) also demonstrated an adaptation or increase in pathotypes of *P. brassicae* able to infect a specific host when exposed to it for many generations.

The effect of different addition times for increasing treatment effectiveness

Adding treatments at different time periods before transplanting did not appear to significantly increase the effectiveness of any of the treatments tested. The only treatment where adding it in advance of transplanting was beneficial to reducing disease levels was the Perlka treatment. This treatment was also added at a lower rate at the same time as adding it to the soil in advance of planting and therefore the effect of timing and the effect of the lower rate cannot be distinguished.

The effect of rates on increasing treatment effectiveness

Adding different rates of microorganisms to the pots in each experiment had an effect on the level of disease control that was gained and also on the plant fresh weight. It was discovered that of the rates tested, low rates of *P. fluorescens* and *P. chlororaphis* were most effective for controlling disease and increasing plant fresh weight. Lower rates of the Bactolife products also had a better effect on plant fresh weight because they were

less phytotoxic. The Perlka treatment was also less phytotoxic when less product was used. More calcium carbonate was used in 2005 than in 2004 but this did not give a better level of disease control. Less LimeX was used in the 2006 experiment than in 2005 (the equivalent of 4 t/ha compared with 10 t/ha) but this lower rate of the treatment still gave complete control against the disease. Spent mushroom compost at 30% (w/w) was better at controlling disease than 10% (w/w) but this higher level of SMC was phytotoxic. These results demonstrated that perfecting the rates of new clubroot control treatments is essential to give an optimum balance of disease control and benefit to plant fresh weight as well as being cost effective.

The effect of treatment combinations at controlling clubroot

Generally, adding treatments in combination to give an additive effect towards disease control was not effective. However, the combination of Perlka and Quickcal completely inhibited disease and may have been due to the extremely high soil pH this combination caused in the soil (pH 8.9). The Borax and calcium combination was also one of the most effective combinations at reducing disease and this may be due to the cell wall strengthening effects that these compounds have. These treatments act in synergy to strengthen cell walls (Webster and Dixon, 1991). Therefore, adding treatments to the soil that have a synergistic action or act on different parts of the *P. brassicae* life-cycle may be the best strategy when developing combinations of treatments for use in the field.

The combination of calcium carbonate and calcium oxide gave complete control. This combination of treatments was tested because it was shown that calcium oxide gave a high increase in soil pH whereas calcium carbonate added a high level of calcium to the soil. This could be a good combination of treatments to use in a clubroot control strategy because high pH and high levels of calcium in the soil have been observed to have a synergistc action in controlling clubroot.

Adding limes in combination with fungicides did not give better disease control than when each treatment was added on its own. This may be because the treatments are both highly effective on their own so there would be no need to add another treatment in combination as it would not improve the optimal level of control that could be achieved by each individual treatment.

Adding treatments in combination suggested that there was a complex interaction between limes fungicides and soil microflora when it came to clubroot control. Until these interactions are understood it would not be beneficial to add various treatments in combination to the soil because the control level could be unpredictable and it would also not be cost effective.

The effect of pH/calcium on clubroot control

The synergism between pH and calcium has been shown to be one of the most important factors in controlling clubroot disease (Webster and Dixon, 1991). The recommended way to control clubroot is to raise the pH of the soil above pH 7.2 (Myers and Campbell, 1985). In these glasshouse experiments many treatments raised the soil pH above pH 7.2 and this did not always give significant disease control. Generally, the treatments that gave complete clubroot control in the soil raised the soil pH to pH 7.3 and above. The exception to this was Shirlan which had a soil pH of 6.8. Shirlan does not act on clubroot through a pH effect but on destroying the respiration apparatus of the resting spores so this may explain that result (Suzuki *et al.,* 1995). Many treatments raised the soil pH above pH 7.3 and did not give complete control. Examples of this are QuickCal which raised the pH to 7.4 but did not give significant control and the combination of Quillaja + calcium carbonate which raised the soil to pH 7.6 and again did not give control.

There was also no specific level of calcium achieved in the soil that was indicative of having a complete controlling effect against this disease. This demonstrated that adding a treatment to the soil in an attempt to reach a certain pH and calcium level that would

be inhibitive towards clubroot would not guarantee control, and that factors other than calcium and pH are involved in control, for example, disease pressure. Webster and Dixon (1991) also showed that calcium and pH operate separately on disease control.

Adding treatments to the outside of module compost to provide clubroot control

Three of the most effective powdered treatments at controlling clubroot disease (calcium carbonate, calcium oxide and shell sand) were added to the outside of the calabrese module compost before transplanting them into the soil to determine whether this strategy could prevent clubroot disease. The reason for doing this was because adding treatments to the outside of modules would reduce the rate of treatment used. This was because in a field environment the treatments are usually spread throughout the soil. It would also concentrate the treatments around the root zone and could create a barrier around the main root system, hopefully reducing infection levels.

The results showed that the calcium carbonate and shell sand treatments were not effective at controlling disease when they were added to the outside of the module compost. Previous researchers have shown that once the plant roots pass out past the treatments in the soil, the roots can become infected by the spores (Tremblay *et al.,* 2005; Cheah *et al.,* 1998). This would therefore suggest that these treatments have to either be in contact with the spores for them to be effective, or have to be in contact with the whole root. The powdered treatments tested in this experiment would only have created a small pocket of high pH and calcium around the module and therefore the roots would have grown out into acidic compost which would then provide optimum conditions for clubroot infection.

The calcium oxide treatment did give a significant reduction in disease level when added to the outside of the module compost. This treatment caused a high rise in pH in the soil that it came into contact with and it also released a lot of heat energy. It would have been expected that adding this treatment so close to the roots would have scorched the

roots and caused the plants to die. This was the case with some plants exposed to calcium oxide, but generally, the plants that did survive were very healthy and this treatment did not cause a significant reduction in plant fresh weight. A possible explanation for the controlling effect of this treatment added in this way was that this treatment caused the roots to initially die when they came into contact with the high pH and heat and therefore the *P. brassicae* spores could not infect the roots. Asano *et al.,* (2000) demonstrated that root hair infection by zoospores occurred most frequently in the section of the root formed one day before inoculation. The roots may have been able to recover after the initial exposure to the calcium oxide, but the *P. brassicae* spores may have already germinated and died in response to the plants being planted and could therefore not infect the newly growing roots.

The high pH caused by this treatment could also have altered the root cell walls by making them stronger because high pH is known to strengthen cell walls (Webster and Dixon, 1988). This would have made it more difficult for *P. brassicae* zoospores to penetrate them. The zoospores may also have been lysed by the heat that the calcium oxide treatment produced.

These experiments have demonstrated that it may be possible to achieve clubroot control by adding treatments to the outside of module compost but that it was more effective to add treatments to the soil that the modules were transplanted into. The treatments should be mixed thoroughly into the soil so that there is an even spread of treatment throughout the soil for the spores and the roots to come into contact with.

Oilseed rape meal timing of addition experiment

An experiment was carried out in the glasshouse to examine the effect of adding oilseed rape meal to the soil at various time-points before planting on clubroot control. Oilseed rape meal contains glucosinolates which have been demonstrated to encourage *P. brassicae* resting spore germination (Walker and Hooker, 1945). Therefore, adding this treatment in advance of transplanting may have encouraged the resting spores to germinate and die in the absence of a host plant hence reducing infection and disease.

The experiment demonstrated that the further in advance of planting OSR meal was added to the soil, the worse the disease. If the spores had germinated in response to the glucosinolates it would have been expected that the further in advance the treatment was added, the less disease would occur because more spores may have germinated and died after a longer exposure to the compounds. Therefore, the glucosinolates in the oilseed rape meal may have been stimulating the spores so that they were ready to complete the life-cycle faster than spores that had not been exposed to this treatment. Researchers in the past who suggested that the glucosinolates stimulated germination may therefore have actually been observing spores that had become more virulent due to the effect of the glucosinolates. This may be why spores exposed to OSR meal for the longest period of time caused the highest level of disease on the plant roots – they had progressed through their life cycle more quickly and formed more galled tissue than spores that had been exposed to the OSR meal for a shorter period of time. The results may suggest that exposure to glucosinolates present in the OSR meal may have turned on biochemical pathways in the spores leading to enhanced virulence. Perhaps a critical enzyme was induced or activated by these substances.

It would not seem likely that the OSR meal added a higher level of spores to the soil through the meal itself being infected with clubroot spores (oilseed rape is also a host of clubroot) because the same amount of treatment was added to the soil at transplanting and this did not increase the clubroot level compared to the control plants but actually decreased it.

The results also demonstrated that plants planted into soil that had been exposed to OSR meal for the longest period of time had a higher fresh weight. This may have been due to the glucosinolates degrading into plant growth hormones such as indole-methyl glucosinolate (an intermediate as well as storage form of auxin in brassicas) (Devos *et al.,* 2005), or it may have been due to the fact that plants with large galls usually had a higher fresh weight as observed throughout the glasshouse trials, possibly as a result of

P. brassicae infection promoting plant growth. The OSR meal may also have added nutrients to the soil that may have affected plant growth.

Chapter 1 reviews the effect of glucosinolates on clubroot, and the literature review in conjunction with this experiment has demonstrated that although OSR meal may not be an effective treatment for clubroot control, glucosinolates in the soil may have an important role to play in the development of clubroot. This enhanced knowledge of factors affecting clubroot development may help in developing ways to reduce disease.

Autoclaving field soil experiment

Field soil that was naturally infested with clubroot was used in a glasshouse experiment to examine what effect, if any, the natural soil biota played in clubroot development. The soil was autoclaved to kill the *P. brassicae* spores and all other soil microorganisms. It was then re-inoculated with varying levels of *P. brassicae* spores. In the field, the soil was conducive to clubroot development.

The results demonstrated that clubroot disease could hardly develop in this autoclaved field soil. At the highest level of spores (10^8 spores/g soil) only two out of the 40 plants planted into the soil showed any sign of disease and this disease was restricted to some slight swellings on the fibrous roots of the plants. The spores were viable because they caused disease on all of the Chinese cabbage plants exposed to them in John Innes no. 2 compost and this also demonstrated that the glasshouse conditions were conducive to disease development because this control tray of plants was planted at the same time as the field soil plants.

These results may suggest that microorganisms play a very large role in the development of clubroot disease because with no other soil microflora present, the disease did not develop. This result reflects the work of Murakami *et al.,* (2002) who demonstrated that field soil that gave a high level of disease showed a lot less disease after it had been autoclaved and inoculated with a high level of spores. The exact mechanism behind this

effect is unknown. There is also a possibility that the autoclaving of the soil could have affected soil structure which may have in turn affected the ability of the spores to infect the plant roots. Murakami *et al.,* (2004) suggested that it was inherent soil properties that gave clubroot control and not the organisms it contained.

Adding increased levels of *P. brassicae* spores to the autoclaved field soil caused the plants to increase in fresh weight and root weight. This may have been because the spores were acting as a carbon source for the plants. The larger amount of spores may also have been producing plant growth hormones or nutrients that the plant could take up and use. It could be possible that some spores had entered the roots and were enhancing the plant metabolism but without showing any sign of disease on the roots.

Soil microflora is known to have an effect on clubroot because it had been observed that resting spores of *P. brassicae* that had been disinfected could not germinate (Castlebury *et al.,* 1994). Asano *et al.,* (2000) also observed that spores prepared in distilled water were not able to germinate as easily as spores prepared in tap water. Buczacki (1983) also demonstrated that there were enhanced *P. brassicae* root hair infections when root hairs were suspended in non-sterile soil. Therefore, some organisms may promote the germination of *P. brassicae* spores in a natural environment.

The *P. chlororaphis* organism tested in this experiment promoted plant fresh weight and plant root weight which would reinforce the fact that this organism is a plant growth promoting organism. When it was added to the re-inoculated autoclaved field soil along with calcium carbonate, the plants had an enhanced fresh weight and root weight which was higher than when either treatment was used alone. This may explain why in the 2006 glasshouse experiment examining the effect of combinations of treatments on clubroot, *P. chlororaphis* reduced the controlling effect of calcium carbonate. This may have been because the *P. chlororaphis* was enhancing plant growth therefore providing more roots for the clubroot spores to enter and enhancing the plants metabolism so that

there were more metabolites for the developing plasmodia to use. The calcium carbonate treatment may not have been able to counteract this effect.

This experiment demonstrated that natural soil microflora may play a large role in clubroot development and discovering which organisms are involved in this mechanism may be a good area for future research.

Conclusions

A wide range of conventional and novel treatments were tested for their effectiveness at controlling clubroot throughout these experiments. The results demonstrated that fungicides that specifically targeted oomycete fungi (Ranman (cyazofamid) and Shirlan (fluazinam)) and treatments such as Perlka which were toxic to the spores were consistently effective at controlling clubroot.

Lime treatments were also confirmed to be very effective at controlling disease with LimeX and powdered calcium oxide being the most effective lime treatments tested. The results also confirmed that the fineness of the limes was critical in achieving good clubroot control. The experiments also demonstrated that increasing soil pH to above pH 7.2 using lime products did not always give complete clubroot control under glasshouse conditions and that to achieve good control, an increase in both pH and calcium may be required.

Calcium containing compounds other than lime such as crushed seashells could be a sustainable way for control as this was a waste product of the fishing industry and was effective at controlling disease in these glasshouse experiments possibly due to the levels of calcium it added to the soil. Bio-processed prawn shells, also a waste product of the fishing industry and spent mushroom compost, a waste product from the mushroom growing industry were effective at significantly reducing clubroot in the glasshouse and could be sustainable treatments for controlling clubroot in the field.

The results of the experiments also suggested that the balance of nutrients in the soil may be important in achieving control. Some nutrients such as potassium may have promoted disease whereas boron and calciummay have prevented it. The correct balance of soil nutrients that prevent clubroot disease would have to be optimised in future research and this knowledge may be useful for practical application in a field environment.

Biocontrol agents were observed to be ineffective at significantly reducing clubroot. An experiment examining the effect of eliminating natural soil microflora in a sample of field soil demonstrated that disease could not occur in the soil despite high levels of viable resting spores being present in the soil. The implications of these glasshouse trials are that they have suggested that the soil microflora may play a major role in clubroot development. It was also observed that the organism *P. chlororaphis* could affect the effectiveness of various treatments at controlling disease. Therefore, in a field environment, the community of soil microflora may explain why treatments can be ineffective at controlling clubroot when they have been shown to be effective in the glasshouse or in different types of soils. The development of a biocontrol agent for use against *P. brassicae* in the soil is therefore something that will require further research.

Some of the treatments that were tested provided insight into factors that affect the *P. brassicae* life-cycle. It was demonstrated that humic acids could promote clubroot and reduce the effectiveness of treatments such as calcium carbonate. Oilseed rape meal also increased the level of disease and suggested that glucosinolates in the soil could enhance spore virulence. Adding a calcium channel blocking treatment such as peppermint oil along with calcium carbonate completely inhibited disease and suggested that there was involvement of calcium channels in either the pathogen or the host in the infection process of *P. brassicae*.

Developing combinations of treatments that could give additive control against clubroot in the field was examined in these experiments. The results showed that certain

combinations of treatments were effective at significantly controlling clubroot (such as calcium carbonate and Borax or Perlka and QuickCal in combination) but that other treatment combinations were no more effective than if each treatment had been added on their own. Some treatment combinations enhanced disease suggesting that the treatments may possibly have the potential to be affected by varying soil factors in the field.

The glasshouse experiments determined that it would be unlikely that adding lime treatments to the outside of module compost would be an effective clubroot control strategy and instead, the limes should be mixed into the soil where the modules are going to be planted to provide a greater level of disease control.

The most effective treatment tested in the glasshouse trials was Perlka added along with QuickCal because this was the only treatment to give complete control and a significant increase in plant fresh weight. For a clubroot control measure to be accepted by growers it would have to increase plant yield as well as reduce the disease level on the roots otherwise it would not be cost-effective to add the treatments to the soil. Many treatments were identified as having a controlling effect against clubroot and a number of these treatments could be considered for field use. However, results observed in glasshouse experiments do not always relate to field environments. In pots, treatments are more concentrated, therefore when translating these glasshouse results for developing control measures for the field, the treatment rates may need to be increased. Soil type, spore load, soil nutrients, soil microflora and weather conditions cannot be controlled in the field unlike in the glasshouse. All of these factors could affect clubroot development and so the same controlling effect that was observed in these glasshouse experiments with some treatments would be unlikely to occur in the field. Future experiments should involve testing the most promising treatments from these glasshouse trials in many different fields under different weather conditions to determine if these treatments could give consistent levels of control commercially.

CHAPTER 4 - CONVENTIONAL AND NOVEL TREATMENTS FOR CONTROL OF CLUBROOT OF BRASSICAS: FIELD EXPERIMENTS

Introduction

It is estimated that clubroot causes £30 million in crop losses in the UK each year (DEFRA, 2003) and worldwide is estimated to account for 10 to 15 percent of all brassica crop losses on an annual basis (Donald *et al.,* 2006). Current control measures largely involve crop rotation to try and reduce the number of resting spores in the soil. However, growers are reluctant to have extended crop rotations because of the high value of brassica crops to their business. Also, resting spores can survive in soil for up to 20 years so the average recommended crop rotation of 6 years is never likely to be fully effective (Tremblay *et al.,* 2000). Adding lime to the soil to raise soil pH is another control method which has been practiced for over a hundred years (Dobson *et al.,* 1983). Despite the extended use of this approach, the actual mechanism by which lime controls clubroot is still unclear and nor is it always effective (Dobson *et al.,* 1983). There are benefits to using lime to control clubroot. It is a cost-effective and readily available substance, it also encourages crumb structure in the soil, encourages decomposition of organic material by promoting microbial action, adds calcium to the soil which is a necessary plant nutrient that encourages good tissue growth and also improves the calcium:magnesium ratio in the soil (Troeh and Thompson, 2005). The repercussions of adding lime to the soil is that excess lime reduces the availability of iron, phosphorous, manganese, boron, zinc, sulphur and potassium (Troeh and Thompson, 2005). The high pH that it causes also reduces the options of crops that could be used in limed fields following its addition. Calcium cyanamide and boron have also been used to control clubroot with limited effect (Dixon and Webster, 1988).

Growers have been reducing all inputs in recent years as they struggle to make any profit (Peter Gladders, pers. com.). In the areas of the UK where intensive brassicas are produced (mainly Lincs, Lancs and Cornwall), if clubroot is a major problem, liming at 2.5 – 5 t/ha is routinely carried out during winter so that ploughing before planting can incorporate it into the soil. Cultivations have been reduced however and therefore, often lime is not mixed into the soil (Nigel MacDonald, pers. com.). Perlka (calcium

cyanamide) is also often added to patches of high spore load at a rate of 400 kg/ha (Peter Gladders, pers. com.). If a patch of land is not intensively cropped with brassicas, spores are only present in small patches and soil pH is greater than pH 7, most growers will do nothing in an attempt to control clubroot (Nigel MacDonald, pers. com).

Given the continuing annual losses, it is clear that the current control measures are not adequate. Existing, partially effective control strategies may still have a role to play in controlling clubroot, but growers increasingly require new cost effective and sustainable solutions that reduce crop losses and sustain vegetable production.

This field based study aims to investigate the efficacy of conventional and novel treatments on the control of clubroot in calabrese. Conventional treatments such as different types of limes and calcium cyanamide were included to allow comparison against existing control measures. Novel treatments included fungicides (either with a broad-spectrum activity or specific activity against oomycete fungi), shell sand, spent mushroom compost, biological control agents, surfactants, rapeseed meal and rye as a bait plant. These treatments were chosen for testing in the field based on the effectiveness of these treatments in preliminary glasshouse trials (Chapter 3). The novel treatments particularly focused on the addition of waste products and biological agents to promote the use of sustainable and natural compounds.

Combinations of treatments such as limes, fungicides and biocontrol agents were investigated in order to elucidate synergistic activity leading to the control of clubroot. Disease reduction (level of incidence and severity), head weight and yield of the plants, and a visual assessment of phytotoxicity were used as a measure of effectiveness of the treatments. This was because the main aim of an effective clubroot control treatment would be one that would reduce the incidence or severity of disease and promote plant growth so that crop yields could be increased. In addition, other factors such as soil pH, extractable calcium level, spore load and race, and weather conditions were also considered in relation to the effectiveness of particular treatments.

Materials and Methods

Choice and location of field trials

Field experiments were carried out near Crail, Fife, Scotland over two years. These sites were chosen because they were known to contain a high level of spore inoculum (Richard Haacker, pers. comm.), and were used commercially for brassica production. Fife is an area of high brassica production and the results achieved in these experiments would be expected to be relevant to the commercial growers of this area. Trial 1 was carried out from 6th June 2005 to 14th September 2005 at Kirkmay farm (NO 604 073) in a naturally infested plot of land that had been intensively cropped with vegetable brassicas over a number of years, the immediately previous crop was calabrese. This land had been intensively treated with lime in previous years. Trial 2 was carried out from 20th July 2006 to 18th October 2006 at Barnsmuir farm (NO 596 062) on a severely naturally infested plot of land that had also been intensively cropped with vegetable brassicas over a number of years, and again, the immediately previous crop was calabrese. For descriptions of the soil types and soil analysis of the fields, see table 4.1. The soil sample used for the soil analyses was taken before any treatments had been added to the soil. The soil was randomly sampled, taking soil from numerous sampling points throughout the trial area to a depth of approximately 10 cm. Approximately 10 kg of soil was sampled in this way. Sub-samples of this soil was used for the various different analyses. The soil analyses were carried out by the Analytical Services Department, Scottish Agricultural College, Edinburgh. For a map of the area where the trials took place see appendix 2, figure 1.

Bait plant testing to provide an indication of the initial spore levels in the soil

Soil that had been randomly collected from the control plots of each field site (see soil pH and extractable calcium analysis section for description of sampling method) before any treatments had been applied to the soil were used in a bait plant test under glasshouse conditions to give an indication of the level of disease that would be expected in the trials. The bait plant test was carried out using the same method that is used for bulking-up of spore inoculum (see Chapter 2). The roots of the Chinese

cabbage were assessed for clubroot severity after six weeks and a disease index was calculated as per chapter 2. Two trays of soil were used for each bait plant test.

Table 4.1. Soil analysis of the two field trial sites

Determination	Result		Units
	Kirkmay 2005	Barnsmuir 2006	
Soil Type	Mineral	Mineral	
Soil Texture	Sandy loam	Sandy loam	
pH	7.5	6.5	
Extractable phosphorous	55.1	37.5	mg/l
Extractable potassium	166	313	mg/l
Extractable magnesium	340	323	mg/l
Extractable calcium	2670	1720	mg/l
Extractable boron	2.4	2	mg/l
Organic matter (LOI)*	7	5.4	%

* Loss on ignition

Experimental design

The 2005 trial tested 12 different treatments in six replications. It was laid out in a rectangle with 14 rows and six columns. There were six blocks, each containing 14 plots in two columns of seven. Plots were 5 m x 1.8 m and blocks were 35 m x 3.6 m. Within each block, each of 12 plots received a different one of the 12 active treatments, and the remaining two plots were used as controls. The reason that two control plots were used was to minimise the effect on the results of the patchy nature of clubroot spores in the field. An alpha-lattice design was used in order to put only one control plot in each column of each block. This design was provided by Dr Chris Theobald, BioSS, Scotland. The soil had been formed into raised beds (figure 4.1) and the total size of the trial area was 30 m x 25.2 m (Appendix 2, Figure 2). The 2006 trial was set out in the same way, also on raised beds, but the total size of the trial area was 30 m x 28.8 m as each of the blocks contained 14 active treatments and two control treatments which meant that each block contained 16 plots in two columns of eight (Appendix 2, Figure 2).

Figure 4.1. Photograph of raised beds used in the field trials showing three strips where treatments were banded

Treatments

Treatments were selected for testing in these field trials because they had been shown to have a controlling effect on clubroot in preliminary glasshouse experiments that had tested a wide range of treatments, both novel and conventional (Chapter 3). Control plots had no treatments added to the soil. For descriptions of the treatments that were added to the soil in the trials see tables 4.2- 4.5. Analysis of the contents of the lime treatments can be seen in tables one to three in Appendix 2. This information was taken from the safety sheets of the products. Analysis of the contents of the spent mushroom compost (SMC) and shell sand treatments can be seen in table 4 in Appendix 2. These treatments were analysed by the Analytical Services Department, Scottish Agricultural College, Edinburgh. The Bactolife DP104 product was activated by adding 0.9 g of the Bactolife DP104 powder to 270 ml of distilled water in a conical flask. To this, 10 g of brown sugar and 4 g of seaweed extract was added. This mixture was aerated at 30^{o}C for 24 hours.

Table 4.2. Description of fungicide treatments added to the soil

Treatment name	Supplier	Description	Active compound (%)
Amistar	Syngenta	Fungicide containing the active ingredient azoxystrobilin	23.1
Ranman	Belchim	Fungicide containing the active ingredient cyazofamid	40
Shirlan	Syngenta	Fungicide containing the active ingredient fluazinam	38.4

Table 4.3. Description of lime treatments added to the soil

Treatment name	Supplier	Description
Calcium carbonate	Buxton lime industries	TruCarb 295 is a fine powdered form of calcium carbonate
Calcium oxide	Buxton lime industries	Calbux 90M is a very fine powdered form of calcium oxide
LimeX	British Sugar	LimeX 70 is a fine powdered form of calcium carbonate which is a by-product of the sugar beet processing industry
QuickCal	PP Products	Flaked calcium oxide

Table 4.4. Description of nutritional treatments added to the soil

Treatment name	Supplier	Description
Perlka	PP Products	A granular form of the fertiliser calcium cyanamide
Shell sand	J & I Anderson	Crushed scallop and whelk shells
Spent mushroom compost	Greenmyre Mushroom Farm Ltd.	This is compost that has previously been used for growing mushrooms

Table 4.5. Description of miscellaneous treatments added to the soil

Treatment name	Supplier	Description
Bactolife DP104	Biotechnica	Commercially available powder containing a mix of 20 different microorganisms such as Bacillus and Pseudomonas species and yeasts
Quillaja 99-99	Biotechnica	Quillaja 99-99 is an extract of quillaja plant saponins
Rye		Rye plants that are grown in the same module tray as the calabrese plants
Unblanched rapeseed meal	Scottish Agricultural College, Aberdeen	A fine powdered meal – a waste product of oilseed rape oil extraction

*ESG-R and ESG-S were coded products tested on behalf of ESG.

Application of treatments

For treatments, the rates of application and timings in the 2005 and 2006 trial, see tables 4.6 and 4.7 respectively. All treatments used in the 2006 trial were applied one day before planting due to non-availability of the site for pre-treatment. In the 2005 trial, treatments 4 – 7 and 11 – 14 were applied to the soil by hand and were incorporated into the bands using a rake. Treatments 3 and 8 were poured onto the soil by hand. Treatments 9 and 10 were incorporated by special tractor-mounted equipment which incorporated the treatments into the soil in a band 20 cm wide by 10 cm deep. In the 2006 trial, treatments 4 – 10, the calcium carbonate and shell sand components of treatments 13 – 15 and treatment 16 were applied to the soil by hand and were incorporated into the bands using a rake. Treatments 3, 12 and the Shirlan components of treatments 13 – 15 were incorporated to a depth of 10 cm by tractor-mounted equipment. Rates were selected based on preliminary work carried out in glasshouse trials (Chapter 3).

Table 4.6. Treatments and application rates in the 2005 trial

No.	Treatment	Rate per field hectare	Rate per treated hectare[1]
1	Control		
2	Control		
3	Bactolife DP104 *	1 kg in 300 l water	3 kg in 900 l water
4	Calcium carbonate *	4 t	12 t
5	Calcium cyanamide (Perlka)	250 kg	750 kg
6	Calcium oxide *	2 t	6 t
7	Shell sand *	2 t	6 t
8	Quillaja	10 l in 1000 l water	30 l in 3000 l water
9	Ranman	4 l in 300 l water	12 l in 900 l water
10	Shirlan	3 l in 300 l water	9 l in 900 l water
11	Spent mushroom compost *	10 t (fresh weight)	30 t (fresh weight)
12	Unblanched rapeseed meal *	1 t	3 t
13	ESG – 1	40 kg	120 kg
14	ESG – 2	40 kg	120 kg

[1] All treatments were applied and incorporated in bands 20cm wide x 10cm deep x 5m long, with the plants placed in a line down the middle of the bands. There were three bands (or lines) per plot. Plots were 1.8m wide x 5m long; therefore the treatments were applied to one third of the plot – which had the effect of concentrating the products threefold within the treated area. Thus the treated rates were three times the field rates.
* Treatment was added 2 weeks before planting.

Table 4.7. Treatments and application rates in the 2006 trial

No.	Treatment	Rate per field hectare	Rate per treated hectare[1]
1	Control		
2	Control		
3	Amistar	3 l in 300 l water	9 l in 900 l water
4	Calcium carbonate	4 t	12 t
5	Calcium carbonate + Shell sand	4 t + 2 t	12 t + 6 t
6	Calcium cyanamide (Perlka)	500 kg + 500 kg*	1.5 t + 1.5 t
7	Calcium oxide	2 t	6 t
8	Shell sand	2 t	6 t
9	LimeX70	10 t	30 t
10	Quickcal	2 t	6 t
11	Rye modules	**	
12	Shirlan	3 l in 300 l water	9 l in 900 l water
13	Shirlan + Calcium carbonate	3 l in 300 l water + 4 t	9 l in 900 l water + 12 t
14	Shirlan + Shell sand	3 l in 300 l water + 2 t	9 l in 900 l water + 6 t
15	Shirlan + Shell sand + Calcium carbonate	3 l in 300 l water + 2 t + 4 t	9 l in 900 l water + 6 t + 12 t
16	Spent Mushroom Compost	30 t (fresh weight)	90 t (fresh weight)

[1] All treatments were applied and incorporated in bands 20cm wide x 10cm deep x 5m long, with the plants placed in a line down the middle of the bands. There were three bands (or lines) per plot. Plots were 1.8m wide x 5m long; therefore the treatments were applied to one third of the plot – which had the effect of concentrating the products threefold within the treated area. Thus the treated rates were three times the field rates.
* 500 kg of calcium cyanamide was added to the soil 1 day before planting, another 500 kg was applied 11 days after planting.
** 2 seeds/module planted 1 week after calabrese seed.

Table 4.8 shows the name of each treatment and the shortened name of the treatment which is used in the graphs throughout this chapter.

Table 4.8. Treatment names and their abbreviations used in the graphs

Treatment	Name/names used in graphs
Amistar	Amistar
Bactolife DP104	Bactolife DP104
Calcium carbonate	Ca carbonate/ CaCO3
Calcium oxide	Ca oxide
LimeX	LimeX
Perlka	Perlka
QuickCal	Quickcal
Quillaja 99-99	Quillaja
Ranman	Ranman
Rye	Rye
Shell sand	Shell sand/SS
Shirlan	Shirlan/Shir
Spent Mushroom compost	SMC
Unblanched rapeseed meal	Rapeseed

Plants

Calabrese cv. Monaco was the test plant in both trials. This was because this cultivar is used commercially in Scotland. Six week old modules (50 cm^3 of soil per module) were provided and planted by the East of Scotland Growers Ltd. The plants were planted deep into the soil (so that just the cotyledons of the plants were showing to a depth of 50 mm) along each of the three rows in a plot with a 12 cm spacing between each plant. This gave 45 plants per plot. The 2005 trial was planted on 17th June 2005, The 2006 trial was planted on 21st July 2006. In the case of the rye module plants, Calabrese cv. Monaco seeds (provided by Syngenta seeds Ltd.) were planted in 50 cm^3 modules filled with Levington compost M3 (Scotts). After the calabrese plants had been grown for 2 weeks, two rye seeds (provided by Suttons Ltd) was added to each module. The plants were grown in a glasshouse under standard conditions (Chapter 2). When the calabrese plant had reached 6 weeks old, these companion plants were planted into the field soil by hand at the same time as the East of Scotland Growers Ltd. planted the rest of the calabrese modules.

Weather data

Maximum and minimum daily temperatures and daily rainfall measurements were obtained from the Leuchars weather station (Leuchars 171, grid reference: 3468 7209). This weather station was the closest weather station in distance from the site of the two field trials.

Soil pH and extractable calcium analysis

Selected plots in the two trials had their soil sampled throughout the trial to determine the pH and extractable calcium levels in the soil. This was an expensive procedure so only treatments that were specifically added to the soil in an attempt to raise soil pH and calcium were analysed. In the 2005 trial, soil samples were collected two weeks before transplanting (just before treatment application), on the day of transplanting (just before transplanting) and at harvest. In the 2006 trial, soil samples were taken just before any treatments were added to the soil and then one day later at transplanting, then 3, 11, 28, 56 and 84 days after transplanting. Samples of approximately 5 kg were randomly taken from within treated rows in the plots. The soil was sampled to a depth of approximately 10 cm using a trowel. Between six and nine sampling points was used for each plot. The samples were submitted to the Analytical Services Department, SAC, Edinburgh where the soil was air dried and milled to pass through a 1mm sieve. An aliquot of 0.01M $CaCl_2$ was added and the pH determined using a pH electrode. To determine extractable calcium content of the soil, the soil was air dried and milled to pass through a 2mm sieve. The extraction was done by the modified Morgans method developed by SAC specifically for Scottish acidic soils (Allen, 1974). Analysis was carried out by Inductively Coupled Plasma Spectrometry (ICP).

Disease severity and yield determination

In the 2005 trial at harvest, yield estimates were calculated by randomly selecting 25 heads of broccoli from each plot (excluding plants from the end of each row in the plot), cutting them to 15 cm in length and weighing them. Twenty five plants per plot were dug up (excluding plants from the end of each row in the plot) and scored for clubroot symptoms on a scale of 0 – 3 where 0 = no infection; 1 = main root healthy, galls present

on one or more lateral roots; 2 = up to one third of the main root galled; 3 = more than one third of the main root galled. A disease severity index (DI) was calculated for each soil based on this scaling. The DI was calculated using the equation from Chapter 2. Phytotoxicity was analysed visually by examining abnormalities in plant growth.

In the 2006 trial at harvest, all heads in a plot were cut to 15 cm and were weighed, excluding the heads on the plants in the rows at the end of each plot. All plants except the plants in the rows at the end of each plot were dug up and scored for clubroot symptoms and a disease severity index was calculated in the same way as the 2005 trial.

European Clubroot Differential (ECD) test to determine spore race

The spore race present in each field soil was determined as per Chapter 2.

Data analysis

The trial was set out in an alpha-lattice design, therefore, the statistical analysis had to take into account the variation between columns in a block as well as between blocks. A spatial analysis of the data which uses knowledge of the row and column position of each plot rather than the block and column was used. Analyses were carried out in Genstat for Windows (7th Edition) (Rothamsted Research) using the residual maximum likelihood (REML) method.

Results

Kirkmay farm, 2005

Disease index 2005

The DI results from the 2005 field trial can be seen in figure 4.2. The two control treatments which had no treatments added to the soil had very similar results and therefore, the results from these plots have been combined to provide an average control treatment result. The control soil had a DI of 65. The rapeseed meal treatment significantly increased the severity of disease in the plants compared to the control plants by increasing the DI to 82. The powdered calcium oxide, shell sand, powdered

calcium carbonate, Ranman and Perlka treatments all significantly reduced the level of disease compared to the control. The most effective treatment at reducing disease was the calcium oxide treatment, reducing the DI to 34. This is a 48% reduction in disease compared to the control plants. The spatial statistical analysis of the disease index results showed that there was no significant difference in disease level between blocks showing that *P. brassicae* spores were evenly spread throughout the trial site.

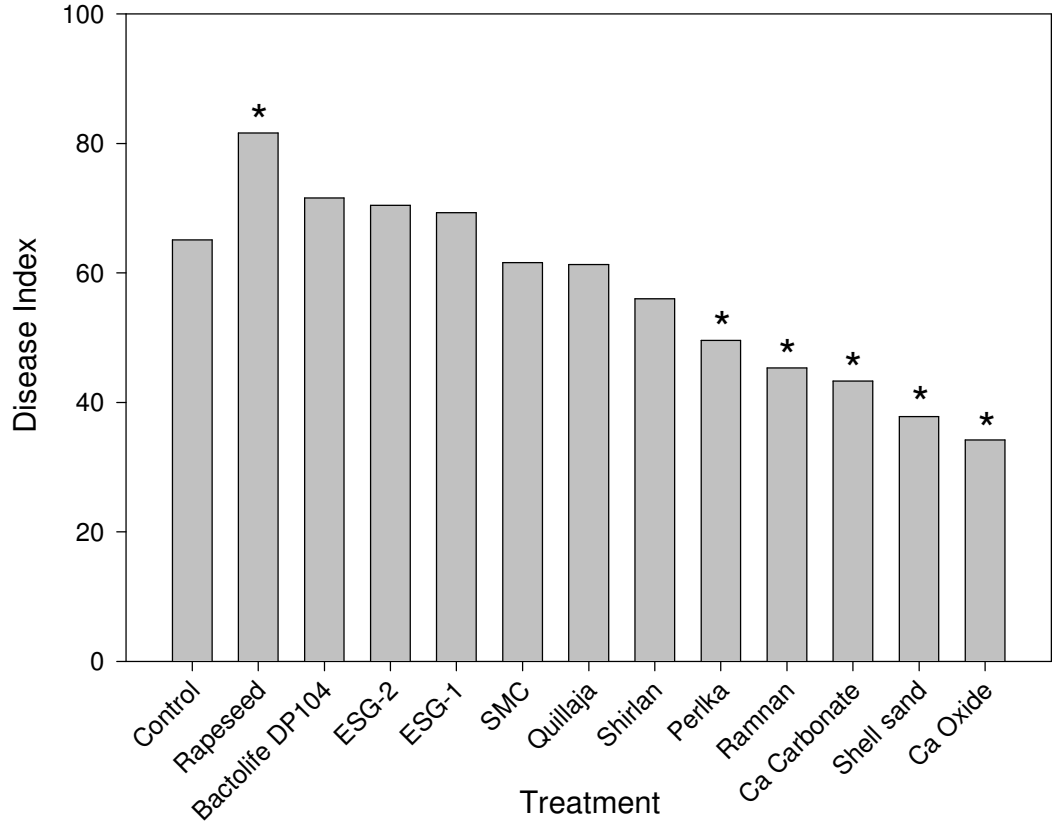

Figure 4.2. Effectiveness of treatments for control of clubroot in calabrese cv. Monaco: 2005 disease index results. Bars show the standard error of the mean (SEM); LSD = 10.53; * P<0.05

Average head weight (yield) 2005

Figure 4.3 shows the results of the head weights from the 2005 trial. The control plots contained calabrese with an average head weight of 338 g. None of the treatments

significantly increased the average head weight compared to the controls. Shirlan significantly reduced the average head weight of the calabrese compared with the control treatment. The average weight of a calabrese head treated with Shirlan was 243 g. This represented a 28% reduction in head weight compared to the controls. Shell sand showed a trend to reduced head weight but this was not significant at the 0.05 level.

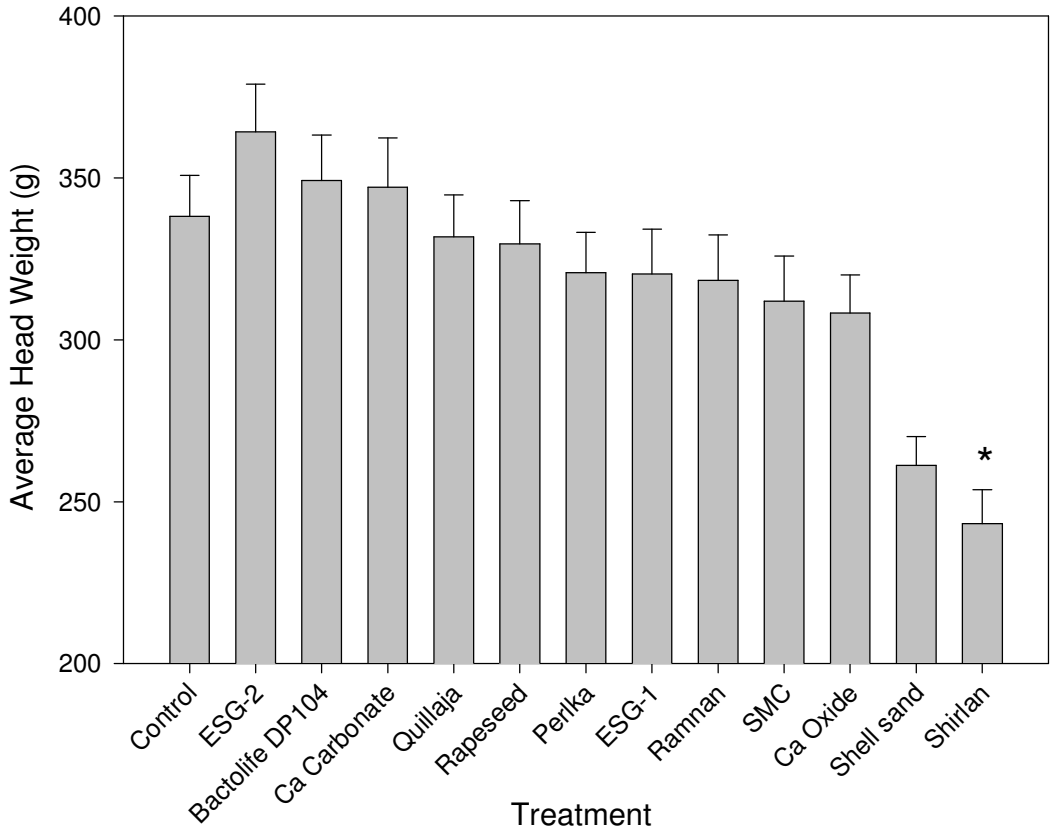

Figure 4.3. Effectiveness of treatments for control of clubroot in calabrese cv. Monaco: 2005 average calabrese head weight results. Bars show SEM; LSD = 86.42; * P<0.05

Phytotoxicity 2005

Phytotoxicity caused by the treatments was not observed on any of the plants in the early growth stages. Three weeks after transplanting, there was no top growth and slight rabbit damage to the crop. The module roots had grown out of the module compost by three weeks after transplanting. At harvest, the Shirlan plots had less heads than any

other plots but only 25 heads from each plot were sampled. The lower number of heads in plots treated with Shirlan could have suggested phytotoxicity.

pH 2005

The lime, Perlka, shell sand and spent mushroom compost treatments were added to the soil 2 weeks in advance of transplanting of the calabrese plants. This was to give time for the treatments to react in the soil and build up soil pH and calcium levels prior to transplantation. The pH of the soil before any treatments were added was between pH 7.4 and 7.5. The pH of the control plot soil and soil treated with lime, Perlka and SMC over time can be seen in table 4.9.

Table 4.9. The pH of control and treated soil at -2, 0 and 12 weeks after transplanting in the 2005 trial

	pH of soil at -2, 0 and 12 weeks after transplanting					
Treatment	- 2 weeks	SEM	0 weeks	SEM	12 weeks	SEM
Control 1	7.4	± 0	7.3	± 0.08	7.1	± 0.04
Control 2	7.4	± 0	7.4	± 0.1	7.1	± 0.06
Calcium carbonate	7.5	± 0.06	8.0*	± 0.04	7.9*	± 0.05
Calcium oxide	7.4	± 0.02	8.3*	± 0.07	8.1*	± 0.06
Perlka	7.4	± 0	7.3	± 0.04	7.5*	± 0.05
SMC	7.4	± 0	7.4	± 0.12	7.1	± 0.09
LSD	0.16		0.23		0.16	

*P<0.05

In both of the control plots, the soil pH decreased over the duration of the trial resulting in a final soil pH of 7.1 at harvest. The calcium carbonate treatment raised the soil pH in the first two weeks to a pH of 8 at transplanting. It then decreased slightly over the next 12 weeks to a pH of 7.9 at harvest. The calcium oxide treatment increased the soil pH to the highest level out of all the treatments tested. It increased the soil to pH 8.3 in the first two weeks after it was added. The soil then decreased in pH to 8.1 at harvest. The Perlka treated plots raised in pH between transplanting and harvest. This was the only treatment to show this trend. The spent mushroom compost treated plots had the same pH and showed the same trend as the control plots showing that the spent mushroom compost had no apparent effect on soil pH.

Extractable calcium 2005

The initial level of extractable calcium in the field soil was 2670 mg/l. The extractable calcium level of the control plot soil and soil treated with lime, Perlka, and SMC over time can be seen in table 4.10. In the first two weeks, the extractable calcium levels in the control plots increased by 10 – 50 mg/l and at harvest, these levels had decreased by 100 – 200 mg/l compared with the level in the soil at the start of the trial.

Table 4.10. The extractable calcium level of control and treated soil at -2, 0 and 12 weeks after transplanting in the 2005 trial

	Extractable calcium (mg/l) in soil at -2, 0 and 12 weeks after transplanting					
Treatment	- 2 weeks	SEM	0 weeks	SEM	12 weeks	SEM
Control 1	2587	± 0	2598	± 59	2467	± 78
Control 2	2587	± 0	2640	± 61	2388	± 67
Calcium carbonate	2623	± 48	10000*	± 698	7582*	± 1199
Calcium oxide	2550	± 50	7732*	± 463	6522*	± 1019
Perlka	2587	± 0	2490	± 84	2607	± 104
SMC	2587	± 0	3172	± 173	2565	± 69
LSD	162		952		1704	

* P<0.05

Calcium carbonate was the most effective treatment at raising soil extractable calcium level in this trial. The level of extractable calcium in the soil two weeks after addition of this treatment was 10,000 mg/l which was the highest level achieved by any treatment measured at any time point. At the end of the trial, the calcium carbonate treated plots had an extractable calcium level of 7582 mg/l. The calcium oxide treatment also raised the extractable calcium level of the soil from 2550 mg/l initially to 7732 mg/l after two weeks to a final level of 6522 mg/l at harvest.

The Perlka treatment had no significant effect on extractable calcium level in the soil, although at harvest, the Perlka treated plots had increased the level of extractable calcium in the soil by 30 mg/l compared with the start of the trial. The spent mushroom compost also had no significant effect on soil extractable calcium level although it increased the level of extractable calcium in the soil by approximately 600 mg/l in the first two weeks after it was added.

Rainfall 2005

The daily rainfall in the Fife area was measured at a weather station in Leuchars which was the nearest weather station to the field trial site. A graph showing the daily rainfall throughout the duration of this experiment can be seen in appendix 2, figure 4. The data shows that the total rainfall over the duration of the field trial was 100.8 mm. The total amount of rainfall that fell in the first two weeks between the addition of some of the treatments and transplanting of the calabrese modules was 27.2 mm. Therefore, 73.6 mm of rain fell between transplanting and harvesting of the plants. This was considered an average rainfall in this area during these months (www.metoffice.gov.uk).

Temperature 2005

The maximum and minimum daily temperature in the Fife area was also measured at a weather station in Leuchars. These figures were used to determine an average daily temperature across the duration of the field trial, the results of which can be seen in appendix 2, figure 5. It shows that there was great fluctuation in temperature over the course of the trial. The average daily temperature across the duration of the field trial was 14.8^{o}C. In the first two weeks of the trial between the addition of some treatments and transplanting of the plants, the average daily temperature was 11.7^{o}C. Between transplanting and harvest, the average daily temperature was 15.4^{o}C.

Bait plant test 2005

A bait plant test on a sample of the field trial soil was carried out in the controlled conditions of a glasshouse to give an idea as to the level of spores present in the soil without the effect of the weather on the development of the disease. The bait plant test showed that the soil gave a disease index of 82 when both replications of the test were combined. This is higher than the disease index of 65 that was seen in the control plots of the field trial.

ECD test 2005

An ECD test was carried out on a soil sample taken from the field trial soil to determine the race of the pathogen that was present in the soil. The pathogen race was determined as 16/31/31.

Barnsmuir farm, 2006

Disease Index 2006

The DI results from the 2006 trial can be seen in figure 4.4. The two control treatments were not significantly different from each other and therefore, the results from these plots have been combined to provide an average control treatment result. The control plots had a high DI of 92. The only treatments that significantly decreased the disease index compared to the controls were Perlka, LimeX and the combination treatment of Shirlan and shell sand. Perlka was the most effective treatment at reducing disease severity on the calabrese plants giving a DI of 61. This was a 33% reduction in disease level compared to the controls. The statistical spatial analysis of the trial showed that there was a difference in disease level between blocks. Blocks in the top right hand corner of the trial had higher levels of disease showing that the level of spores present in this soil was higher in the top right hand corner of the trial compared to the rest of the trial.

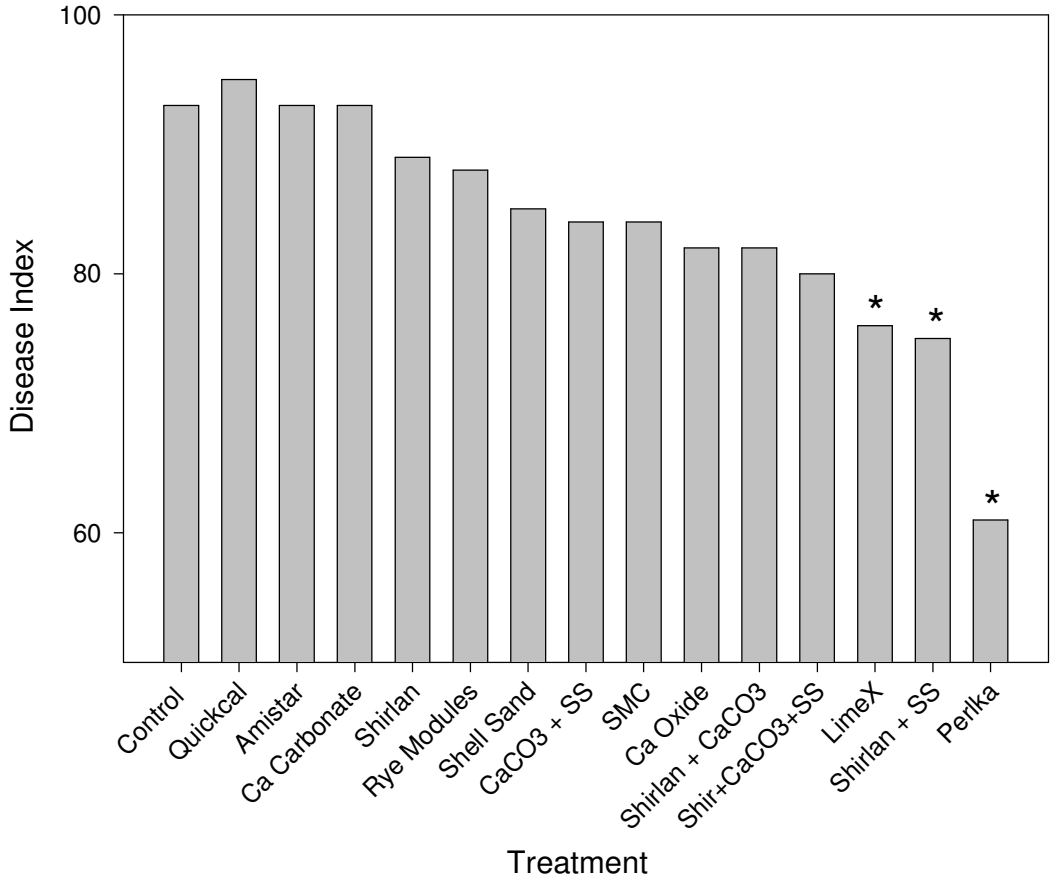

Figure 4.4. Effectiveness of treatments for control of clubroot in calabrese cv. Monaco: 2006 disease index results. LSD = 13.62; * P<0.05

Average head weight 2006

Figure 4.5 shows the average head weight of the plants in the 2006 trial. The control plots had an average head weight of 221 g. LimeX, Perlka and spent mushroom compost significantly increased the average head weights to 290 g, 288 g, and 269 g respectively. This is around a 20% increase in average head weight compared to the controls. No treatments decreased the average head weight compared to the controls. The statistical spatial analysis of the trial showed that there was a difference in average head weight between blocks. Blocks in the top right hand corner of the trial had lower average head weights.

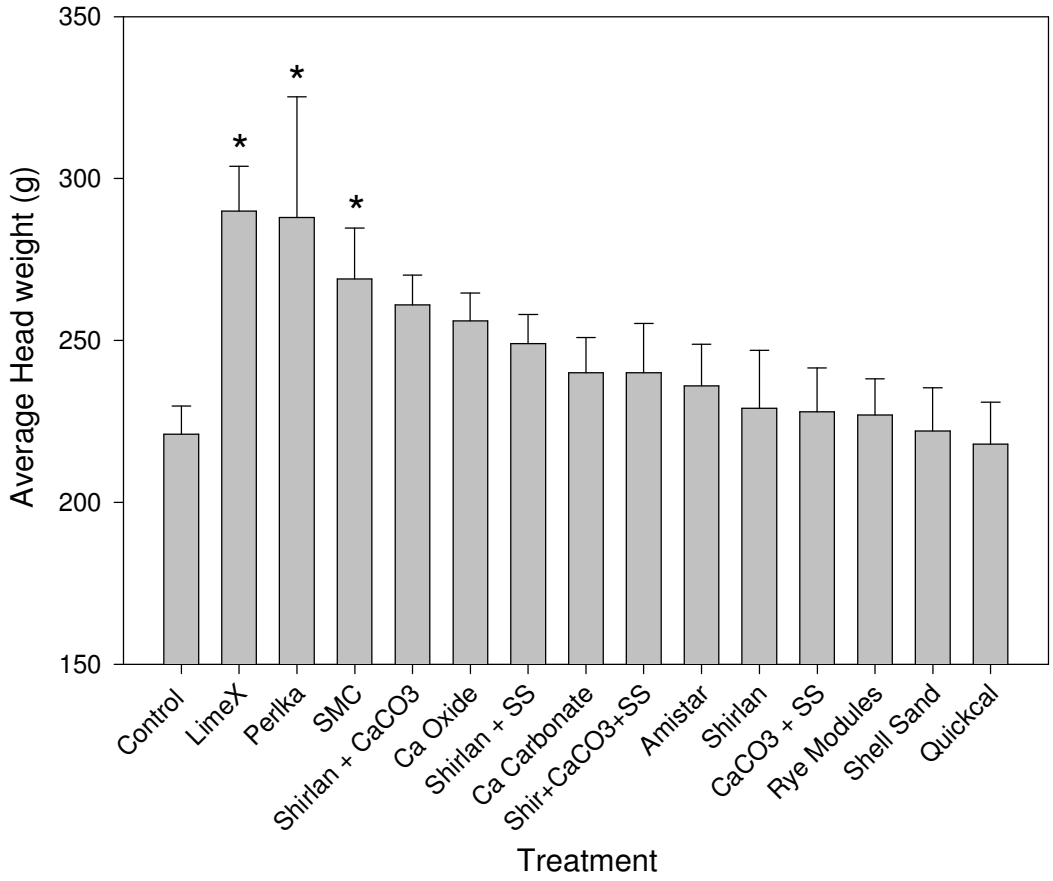

Figure 4.5. Effectiveness of treatments for control of clubroot in calabrese cv. Monaco: 2006 average head weight results. Bars show SEM; LSD = 42.73; * P<0.05

Calabrese yield 2006

The total calabrese yield for each treatment was calculated by combining all of the head weights of the calabrese exposed to a treatment together (excluding the buffer plants in the end rows of each plot). These results can be seen in figure 4.6. Amistar, Perlka, LimeX, SMC and the combination treatment of Shirlan and calcium carbonate significantly increased the yield of the calabrese compared to the controls. None of the treatments significantly decreased the yields compared to the controls. The yield results show that although Amistar and the combination of shell sand and calcium carbonate did not increase average head weight, they increased overall yield.

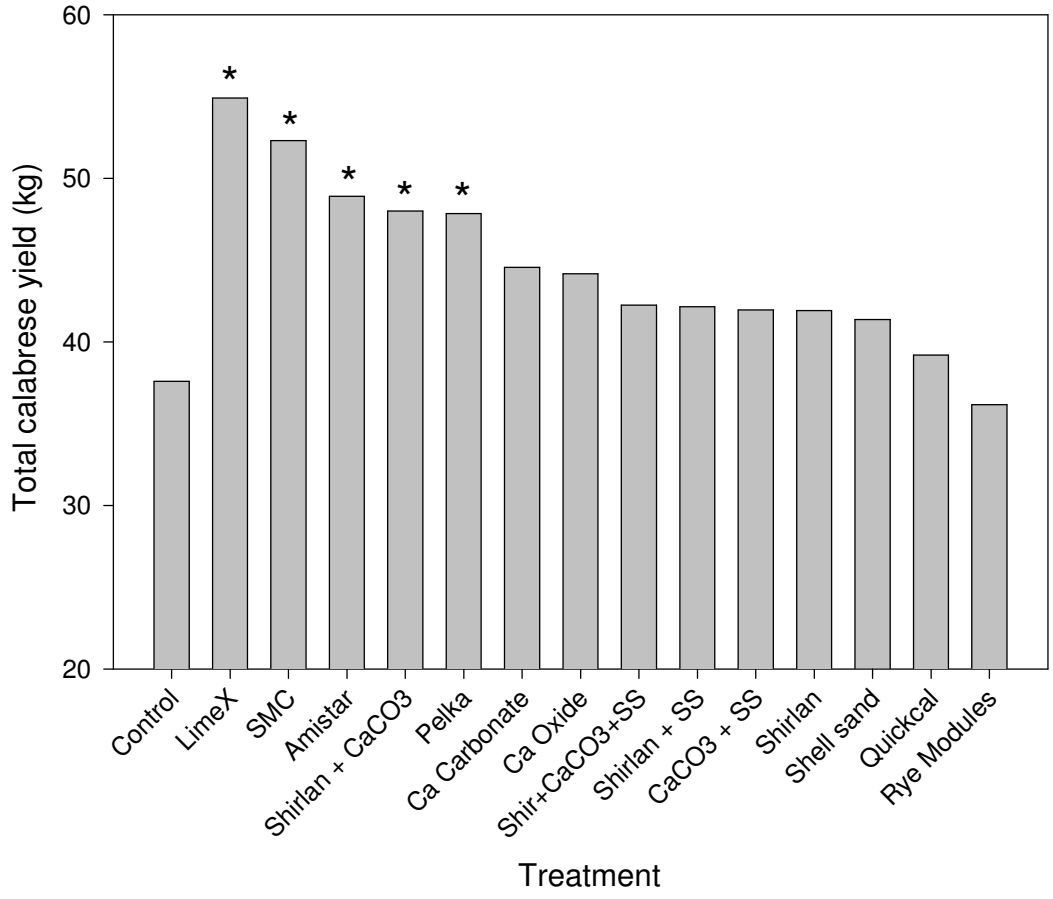

Figure 4.6. Effectiveness of treatments for control of clubroot in calabrese cv. Monaco: 2006 total calabrese yield results. LSD = 10.08; * P<0.05

Phytotoxicity 2006

Phytotoxicity caused by the treatments was not observed on any of the plants in the early growth stages. Two weeks after transplanting, there was little rooting and slight bird damage to the crop. At harvest, all of the heads in a plot were sampled. There was no significant difference between the number of heads measured in any plot with the exception of the Amistar plot which yielded a significantly greater number of heads than the control plots.

pH 2006

The pH of the field soil was pH 6.5. The effect of the treatments on soil pH can be seen in table 4.11. The pH analysis of the control plots showed that 3 days after transplanting, the pH in the control plots had increased to pH 6.6. The pH of the control plot soil fluctuated slightly throughout the duration of the trial but there was an overall decrease in pH with the pH of the soil at harvest between pH 6.2 and 6.3.

Table 4.11. The pH of control and treated soil on selected days after transplanting in the 2006 trial

	pH of soil on selected days after transplanting						
Treatment	-1	0	3	11	28	56	84
Control 1	6.5	6.5	6.6	6.3	6.5	6.2	6.2
Control 2	6.5	6.6	6.6	6.4	6.5	6.1	6.3
Calcium carbonate	6.5	7.4*	7.5*	7.4*	7.5*	7.3*	7.4*
Calcium oxide	6.5	8.3*	8.1*	7.6*	7.7*	7.4*	7.6*
LimeX	6.5	7.5*	7.8*	7.7*	7.8*	7.5*	7.7*
Perlka	6.5	6.8*	6.8	6.9*	7.3*	7.3*	7.4*
QuickCal	6.5	6.8*	7.3*	6.5	6.8*	6.5*	6.8*
Shell sand	6.5	6.8*	7.0	6.9*	6.9*	6.8*	6.9*
SMC	6.5	6.9*	6.9	6.7*	6.9*	6.6*	6.7*
LSD	0	0.12	0.43	0.16	0.15	0.23	0.19

*P<0.05

The SEM figures for this table can be seen in Appendix 2, Table 5.

The calcium carbonate and LimeX treatments raised the pH of the soil very quickly – to pH 7.4 and 7.5 respectively in the space of a day. When the soils were sampled at three days after transplanting, the highest pH measurement was observed for these limes compared with any other sample timings throughout the duration of the trial. LimeX raised the pH of the soil more than the calcium carbonate treatment throughout the duration of the trial. The calcium carbonate and LimeX treated soil increased the pH of the soil to pH 7.4 and 7.7 respectively at harvest. These treatments had a significant effect on soil pH throughout the trial.

The powdered calcium oxide treatment raised the pH of the soil to the highest pH of any of the tested soil at any time point – from pH 6.5 to pH 8.3 in the space of one day. The QuickCal treatment raised the pH of the soil in the space of one day to pH 6.8. The powdered calcium oxide treated soil reduced in pH after the calabrese modules were added to the soil. At harvest, the pH of the soil treated with powdered calcium oxide was 7.6. The QuickCal treatment increased the pH of the soil to pH 7.3 three days after transplanting. There was then a very rapid decrease in pH of soil treated with this treatment so that by 11 days after transplanting the pH was 6.5. The pH of the soil treated with QuickCal then increased in pH, and fluctuated throughout the remainder of the trial. It gave a significant increase in pH in the latter stages of the trial compared with the control soil.

The Perlka treatment significantly increased the soil pH within one day of it being added to the soil from pH 6.5 to pH 6.8. At 11 days after transplanting, another application of Perlka was made to the soil. The pH of the soil treated with Perlka increased throughout the trial reaching a final pH of 7.4 at harvest. The shell sand treatment increased the pH of the soil by the same amount as the Perlka treatment did (to pH 6.8) in the first day after addition. Soil treated with this treatment reached pH 7 three days after transplanting. At 56 days after transplanting, soil treated with shell sand had increased to a pH of 7.4. The spent mushroom compost increased soil pH from pH 6.5 to pH 6.9 within 1 day. The pH level of the soil treated with this treatment fluctuated between pH 6.6 and 6.9 in the course of the trial. At harvest, soil that had been treated with spent mushroom compost had a pH of 6.7.

In general, the pH results show that pH increased at the three day sampling point to the maximum levels revealed. The pH then decreased by the eleven day sampling point after transplanting (with the exception of Perlka). By 28 days post transplanting pH increased in all treatments, pH then decreased by 56 days post transplanting and at harvest, pH had increased in level compared with sampling day 56. The effect that each treatment had on soil pH was similar to the effects seen in the 2005 trial.

Extractable calcium 2006

The extractable calcium level of the soil previous to the addition of treatments was 1720 mg/l. In the control plots (Table 4.12), the extractable calcium level stayed the same in the first day. Between transplanting and three days after transplanting, the control plots had an increased extractable calcium level of approximately 50 mg/l. This was a small but non-significant increase. At harvest, extractable calcium was decreased by approximately 200 mg/l compared with the pre planting level.

Table 4.12. The extractable calcium level of control and treated soil on selected days after transplanting in the 2006 trial

Treatment	Extractable calcium level (mg/l) of soil on selected days after transplanting						
	-1	0	3	11	28	56	84
Control 1	1868	1868	1910	1848	1895	1747	1663
Control 2	1828	1828	1885	1867	1887	1642	1732
Calcium carbonate	1848	3755*	6263*	5067*	5223*	4230*	5213*
Calcium oxide	1848	5722*	7138*	5288*	7063*	4843*	4472*
LimeX	1848	3502*	6543*	5585*	7147*	5023*	4627*
Perlka	1848	1908	1897	1927	2292	2275	2623
QuickCal	1848	2050	2800	2052	2283	1985	2340
Shell sand	1848	2842*	4080*	3495*	3667*	3460*	2420
SMC	1848	2398	2398	2343	2550	2347	2225
LSD=	0	838	1208	1054	1213	1160	957

*P<0.05

The SEM figures for this table can be seen in Appendix 2, Table 6.

The extractable calcium levels for the calcium carbonate and LimeX treatments followed essentially the same patterns (Table 4.12). The only significant difference was observed on sample day 28 where the LimeX treated soil had the highest extractable calcium level out of any of the treatments tested on that date. At harvest, calcium carbonate had increased soil extractable calcium level to 5213 mg/l and LimeX had increased it to 4627 mg/l.

The effect of the different types of calcium oxide treatment to the soil on soil extractable calcium level can also be seen in Table 4.12. The QuickCal treatment had no significant effect on soil extractable calcium levels, although a slight but non-significant increase in soil extractable calcium was observed on day 11. The powdered calcium oxide gave the highest increase in the extractable calcium level within one day out of any of the treatments tested. At 3 days after transplanting, the powdered calcium oxide treated soil had the highest extractable calcium level of any treatment tested at any time point (7183 mg/l). The level of extractable calcium in the soil treated with powdered calcium oxide at harvest was 4472 mg/l. The level of extractable calcium in the QuickCal treated soil at harvest was 2340 mg/l.

Perlka, shell sand and spent mushroom compost effects on soil extractable calcium level can be seen in table 4.12. The Perlka treated soil showed a very small rise in extractable calcium level in the first day after addition. This was followed by a stable period before rising. It was the only treatment tested that caused the extractable calcium level in the soil to increase higher than the level it provided at the day 3 sampling point. Spent mushroom compost increased the level of extractable calcium in the soil by approximately 600 mg/l in 24 hours but this was not a significant rise compared with other treatments. This level stayed reasonably stable across the course of the trial. The shell sand treatment increased the extractable calcium level in the soil by around 1000 mg/l within one day. This large early rise was followed by a slow tail off in extractable calcium level. The Perlka, SMC and shell sand treatments all had a similar rate of extractable calcium at harvest which was not significantly different to the control plot levels.

In general, the results show that 3 days after transplanting (4 days after addition of treatments) is when the highest level of extractable calcium levels were recorded in the soil. After this point, extractable calcium decreased until 11 days after transplanting and then increased again, following the same patterns as the soil pH level for each treatment.

The effect that each treatment had on extractable calcium was similar to the effects seen in the 2005 trial.

Rainfall 2006

The daily rainfall in the Fife area was measured at a weather station in Leuchars. The results can be seen in Appendix 2, Figure 6. The data shows that the total rainfall over the duration of the field trial was 173.8 mm. The total amount of rainfall that fell between the addition of the treatments and 3 days after transplanting was 0 mm. Between 3 and 11 days after transplanting, there was 11.36 mm of rainfall. Between 11 and 28 days after transplanting there was 14.2 mm of rainfall. Between 28 and 56 days after transplanting there was 84.8 mm of rainfall and between 56 days after transplanting and harvest there was 71.2 mm of rainfall. The total rainfall of 173.8 mm over the duration of the 2006 trial is considerably higher than the level that fell during the duration of the 2005 trial which was 108 mm.

Temperature 2006

The maximum and minimum daily temperature in the Fife area was also measured at a weather station in Leuchars. The average daily temperature was calculated from these measurements and the results can be seen in Appendix 2, Figure 7. The average daily temperature across the duration of the field trial was 14.8^{o}C. The average daily temperature between the addition of the treatments and 3 days after transplanting was 19.1^{o}C. Between 3 and 11 days after transplanting, there average daily temperature was 18.6^{o}C. Between 11 and 28 days after transplanting there average daily temperature was 20.9^{o}C. Between 28 and 56 days after transplanting the average daily temperature was 15.4^{o}C and between 56 days after transplanting and harvest the average daily temperature was 13^{o}C. The 2005 trial also had an average daily temperature of 14.8^{o}C but was colder in the first few weeks of the trial compared to the 2006 trial temperature.

Bait plant test 2006

A bait plant test on a sample of the field trial soil was carried out in the controlled conditions of a glasshouse to give an idea as to the level of spores present in the soil without the effect of the weather on the development of the disease. The bait plant test showed a disease index of 92. This is the same disease index of 92 that was seen in the control plots of the field trial and higher than the level seen in the bait plant test carried out on the 2005 field soil, but this was not significantly higher.

ECD test 2006

An ECD test was carried out on a sample of the field trial soil to determine the race of the pathogen that was present in the soil. The pathogen race was determined as 16/02/30 which was different to the spore race determined to be present in the 2005 soil.

Discussion

The most effective treatments at controlling clubroot disease in the field trials were the conventional clubroot treatments of lime, Perlka and fungicides. The trials support similar work done by other researchers in the same topic – essentially, treatments used to control clubroot do not act in a consistent way to control the disease. More treatments controlled clubroot disease in the 2005 trial compared to the 2006 trial and the possible reasons for this are discussed. Some of the more novel treatments tested for clubroot control were not as effective as limes, Perlka or Ranman and Shirlan and the possible reasons for this will also be discussed.

Control plots showing differences between the two field trials

There could be many reasons for the higher amount of disease in the control plots in the 2006 trial compared to the 2005 trial. A bait plant test carried out on the two field soils in controlled glasshouse conditions showed that there may have been greater disease pressure in the 2006 trial compared with the 2005 trial. The 2005 bait test showed a DI of 82 and the DI of the field control plots was 65. This may suggest that bait plant tests that are carried out in the glasshouse on field soil are not fully indicative of disease

levels that will be seen in the field. The conditions such as temperature and soil moisture in the glasshouse may have been more conducive to disease development than in the field in this case.

The two trials also differed in spore race as seen by the European Clubroot Differential (ECD) tests. Different spore races can vary in their virulence towards host plants (Voorips, 1995) and this may account for the differences between the trial years and sites. The pre-planting soil analysis carried out on the two field soils also differed between the two sites. Both soils were of the same texture (sandy loam) but the pre-planting pH of the 2005 trial (pH 7.5) was higher than the pre-planting pH of the 2006 trial (pH 6.5). A pH of 7.2 or greater is generally thought of as being the optimum pH for controlling clubroot (Colhoun, 1953). Although the pH of the 2005 soil was above pH 7.2, clubroot was not completely controlled in this soil, although the high initial soil pH may have prevented the disease development to some extent. The pH of 6.5 in the 2006 trial soil favours clubroot development (Campbell *et al.,* 1985) and this may be why there was a high level disease in the control plots in this trial compared with the 2005 trial.

The initial extractable calcium content of the soil of the 2005 soil was also higher than in the 2006 trial. Calcium is known to have a controlling effect on clubroot (Myers and Campbell, 1985) and therefore the 2005 soil was less likely to be optimal for clubroot development than the 2006 soil. Boron, another element thought to have an effect on controlling clubroot (Dixon and Webster, 1988) was also higher in the 2005 soil compared with the 2006 soil providing another possible explanation as to why there was less disease in the 2005 trial compared to the 2006 trial.

The weather differences between the two field trials may provide clues as to why higher disease was seen in the control plots of 2006 compared with 2005. The 2005 trial took place earlier in the year than the 2006 trial but the average daily temperature across the duration of both trials was the same. Thus, plants in the 2006 trial may have

experienced temperature stress at an early growth stage and were therefore susceptible in the early stages of infection resulting in greater disease severity at the end of the trial. The effect of light and temperature is also greatest in disease development within the first two weeks of infection (Jones *et al.,* 1982) and so the temperature in the 2006 trial may have been enhancing the severity of the disease.

The 2006 trial was also not irrigated and was planted during a severe drought. There was no rainfall in the first three days after transplanting in the 2006 field trial and this could mean again that the 2006 plants were stressed when they were first planted and were therefore more susceptible to infection. The 2005 trial had a total rainfall of 100.8 mm across its duration. The 2006 trial had a total rainfall of 173.8 mm. This is around 70% more rainfall than in the 2005 trial. Warm, wet conditions are known to be optimal for allowing clubroot symptoms to develop (Thuma *et al.,* 1983) and this may be another clue as to why the disease was more severe in the 2006 trial compared with the 2005 trial.

The control plot results also showed that the pH of the soil decreased over the trial duration. This was the same in both trials despite the differences in the initial pH of both of the soils. This reduction in pH may be the natural pH reduction in a calabrese season. Scottish soils are naturally very acidic so a fall in pH during a trial is inevitable (Allen, 1974). The pH fluctuations in the control plots were more evident in the 2006 trial where soil was sampled at more time points than in 2005. It showed that there may be a slight increase in soil pH within the first three days after planting calabrese plants into the soil. It is usually seen that brassica plants acidify the rhizosphere to increase available phosphates (Hedley *et al.,* 1982). Therefore the alkalisation seen in these results could be due to the plant excreting substances (such as the secondary metabolites alkaloids) into the soil as a response to the challenge by *Plasmodiophora brassicae* spores. Takahashi *et al.,* (2006) observed that Chinese cabbage alkalised the rhizosphere in response to *P. brassicae* spores under glasshouse conditions. An alkalisation of the rhizosphere by *Arabidopsis thaliana* in response to *P. brassicae* has also been shown by

Fuchs and Sacristan (1996) in glasshouse conditions. The results of the field trial could show that this response also occurs in the field environment. The pH of the control plots fluctuated over the duration of the 2006 trial and this could be as a result of rainfall. Rainfall may activate volatile substances in the soil (for example, secondary metabolites such as glucosinolates) which could vary its pH, or it could be enhancing microbial activity in the soil and these microbes could be producing pH altering substances (Buscott and Varma, 2005).

The extractable calcium levels measured in the control plots of the two trials showed that over the duration of the field trial, the extractable calcium level decreased by around 200 mg/l of extractable calcium. This could be due to leaching or plant uptake. In the 2006 trial it was shown that the control plots increased in extractable calcium level by around 50 mg/l within the first 3 days after transplanting. This calcium could be being released by plants, perhaps as an indication of a hypersensitive response taking place (Xu and Heath, 1998), or by microorganisms in response to the plants, or the *P. brassicae* spores are eliciting this response. This is because an increase in calcium ions in the soil would alkalise the rhizosphere and the alkalisation of the rhizosphere in response to *P. brassicae* has been observed by Takahashi *et al.,* (2006) and Fuchs and Sacristan (1996).

The effect of calcium carbonate containing compounds on clubroot control in the field trials

Calcium carbonate lime has been used for many years as a clubroot control measure because the increase in pH and calcium that it provides to the soil is known to have a controlling effect on *P. brassicae* (Webster and Dixon, 1991). In the 2005 trial, calcium carbonate was one of the treatments that significantly reduced disease on the plants compared with the controls. The same effect in the 2006 trials was not seen despite the fact that the same amount of the product was used. It had no significant effect on head weight or yield in either trial. This result is different to the observations of Fletcher *et al.,* (1982) who showed that calcium carbonate increased plant head weights. Murakami

et al., (2002) used calcium carbonate in their trials and found that they observed the same level of disease control no matter how much of the product was added. Calcium carbonate raised the pH of the soil from pH 7.5 to pH 8 in the space of two weeks in the 2005 trial and from pH 6.5 to pH 7.5 in the space of two weeks in the 2006 trial. These results would suggest that there is a limiting level to how much soil pH can be raised using calcium carbonate and this may explain why Murakami *et al.,* (2002) could not find improved control by adding more calcium carbonate to the soil.

The calcium carbonate treatment raised the pH level in the soil rapidly within the first 24 hours after addition in the 2006 trial. The fineness of this powder is likely to be the reason for this. It also raised the soil extractable calcium levels very rapidly within the space of 24 hours. The highest level of extractable calcium in the soil treated with this treatment was observed at 2 weeks after addition in both the 2005 and 2006 trials. At transplanting in the 2005 trial, it provided the highest calcium level of any of the treatments. It did not have such a significant effect at increasing extractable calcium level in the soil in the 2006 trial. However, the pH in the 2006 trial was lower and calcium becomes more available as pH increases (Myers and Campbell, 1985). The lower level of calcium provided by this treatment at transplanting in the 2006 trial compared with the 2005 trial may provide a clue as to why there was a difference in the control achieved by this treatment in the two trials. The inconsistencies observed in the two trials carried out in this study may result from the differences in timing of addition of this treatment between the two seasons (two weeks and one day in advance of transplanting, 2005 and 2006 respectively).

Although the pH was raised to its highest level one day after addition of the treatment, it may be the case that clubroot spores have to be exposed to a high level of pH for a longer period of time than one day to effectively control the disease. This hypothesis would agree with the findings of Colhoun (1953) who also discovered that exposing spores to high pH levels for a long period of time reduced their infectivity. Therefore, transplanting time in relation to treatment addition may be important in developing

consistently effective clubroot control measures. Although lime application is often recommended at least 6 weeks before planting (reflecting the time required for lime to be distributed and solubilised under field conditions) (Dobson *et al.,* 1983), it may not be necessary as good control can also be obtained with application one day before planting (Campbell *et al.,*1985). This was also observed in glasshouse and field experiments (chapters 3 and 4). This infers that calcium and high pH may have little fungicidal effect on resting spores but may affect pathogen penetration or development in the host. In practice, it may be better to apply lime close to sowing to have the greatest clubroot control. Murakami *et al.,* (2002) demonstrated that clubroot was significantly lower when lime products were thoroughly mixed into the soil 2 weeks before sowing compared with 4 weeks. Donald *et al.,* (2006) also suggested that early application of calcium was required to protect young transplants from infection. Larson and Walker (1944) showed that as the interval after treatment with calcium hydroxide increased, soil pH became more acid and the percentage of infected plants increased. They also observed that adding lime to the soil three days before planting gave slightly better disease control than adding the lime months in advance of transplanting. In a Californian field trial carried out by Campbell *et al.,* (1985), a reduction in exchangeable calcium of 140 mg/kg in field soil over the course of a year was probably due to leaching during winter rains. Therefore, to keep the calcium and pH level of the soil high, lime should be applied close to sowing/transplanting. Single applications of calcium carbonate at 10 t/ha or 20 t/ha controlled clubroot for up to 3 years in the soil in an experiment carried out by Fletcher *et al.,* (1982). There were no marked differences if the calcium carbonate was applied as a single or a split application. Fletcher therefore concluded that lime needed to be applied at a high rate to be effective. Takahashi *et al.,* (2006) saw an alkalisation of root culture medium in resistant cultivars two days after they were inoculated with *P. brassicae*. Early responses may contribute to strong resistance and perhaps adding lime in the early days of infection mimics the effect of the resistant plant response. High pH however could give issues to other crops used in a rotation and may be limiting to certain break crops from vegetables. Banding of lime treatments was also observed to be effective at providing good disease control. This

gave the required level of control while at the same time reduced the amount of product required, and therefore cost, by about two thirds.

In the 2006 trials it is also possible that disease levels overwhelmed any treatment effects and despite a reduction in spore loading, disease expression was the same. This has been observed by Jones *et al*., (1982) who demonstrated that even a small number of spores entering the root cortex can cause a high level of disease.

Calcium carbonate as a control agent has also been shown to present inconsistent effects in other field trials (Murakami *et al.*, 2002), (Fletcher *et al.,* 1982) and therefore may not be the most effective treatment for clubroot control.

The LimeX treatment which is another form of calcium carbonate was only tested in the 2006 trial and was tested in order to facilitate effective comparisons between different forms of lime compounds. LimeX significantly reduced the disease severity in the 2006 trial and also significantly increased head weights and yield of the calabrese compared with the control. It was one of the most effective treatments tested in this study. LimeX at the rate used was slightly better at increasing the pH of the soil compared with the calcium carbonate treatment. This may be due to the fact that there was more LimeX added to the soil compared to the calcium carbonate due to the differences in neutralising value between the two. LimeX is also in the form of a very fine powder and is therefore also very reactive in the soil (www.limex.co.uk) which is why the pH increased so quickly with this treatment. In addition, LimeX was also very effective at increasing the soil calcium levels which is known to play a part in clubroot control. LimeX not only contains calcium carbonate but also contains more nutrients than quarried calcium carbonate such as phosphates, magnesium and sulphates (www.limex.co.uk). These additional nutrients may have promoted plant nutrition, improving plant health and capability to withstand attack by the pathogen. LimeX is essentially a waste product. Therefore, using this treatment for clubroot control would be of potential benefit to both brassica growers and British sugar industry. Its use as a

control treatment in horticulture would have environmental implications because it would not have to be added to landfill. On the basis of glasshouse trials (Chapter 3) and the 2006 field trial, LimeX appears to have benefits in clubroot control above the effect of using standard lime. This treatment was effective in the 2006 trial which had more optimum soil and weather conditions for clubroot development than the 2005 trial, and so these results show that even under optimum conditions for clubroot development, adding LimeX to the soil could be a good method for reducing disease severity.

The effect of different forms of calcium oxide on clubroot control in the field trials

In the 2005 trial, powdered calcium oxide was the most effective treatment at reducing clubroot disease severity but it had no effect on head weight or yield. There were no visual signs of phytotoxicity caused by this treatment. Calcium oxide gave the highest soil pH increase in 2005 causing the soil to reach a pH of 8.3 in two weeks and it was also effective at increasing soil calcium levels. As already indicated, high pH and calcium levels are known to have a controlling effect on clubroot and this could explain why this treatment was very effective at controlling disease severity in 2005. However, the same powdered calcium oxide treatment used in 2006 had only a slight but statistically non-significant effect on controlling the disease. The powdered calcium oxide treatment was also very effective at raising the soil pH in the 2006 trial. It raised the soil from pH 6.5 to pH 8.3 within the space of 24 hours which was the highest pH out of any of the treatments tested. The likely explanation for the failure to observe an effect on the control of the disease in the 2006 trial lies in the inability to apply the treatment until 24 hours before transplanting and as a result, the spores may not have been in contact with the enhanced pH for a long enough period to prevent further development of the spores. Calcium oxide provides heat energy by the formation of calcium hydroxide when it comes into contact with water. The high pH rise and the heat caused by the addition of calcium oxide to the soil may explain why it has an effect on clubroot disease. The heat may destroy the spores by denaturing proteins and therefore destroying the cell wall, and a large increase in pH could lyse spore membranes (Heinonen-Tanski *et al.,* 2006).

The ability of the powdered calcium oxide to raise soil pH so quickly may be due to the physical nature of the material used, fine powder with large surface area making it highly reactive. However, fine powders are not really acceptable to growers for health and safety reasons as it blows in the wind and is a respiratory hazard (Richard Haacker, ESG, pers. comm.). As powdered calcium oxide is not widely accepted for use by growers, a flaked form of calcium oxide called QuickCal which is more easily spread in the soil was used in the 2006 trial. Unfortunately, it was the least effective treatment at controlling disease and increasing head weight. It did increase soil pH within one day after addition but this was less of a pH increase as caused by the powdered calcium oxide. The pH of the soil following treatment with QuickCal fluctuated over the course of the trial. This may reflect the effect that rainfall has on the flaked calcium oxide. Large flakes were found in the soil throughout the trial and as rain fell, calcium hydroxide would be produced, increasing soil pH. The pH would then decrease over time until more rain fell. QuickCal did not enhance soil calcium to the same level as the powdered form. The most likely cause for this is because of the surface area of the flaked calcium oxide. The flakes have a reduced surface area, and the localisation of action (heat, pH and calcium) would ensure that contact with spores would be reduced, thereby resulting in limited efficacy of the treatment. These results show that the fineness of the grinding of the lime is likely to be important to the pH and calcium levels that are reached in the soil and which in turn, may influence the value of limes as a control agent. This confirms work carried out by other researchers that limes with a finer particle size are better at providing clubroot control (Dobson *et al.*, 1983) (Murakami, *et al.,* 2002) but this research was carried out in America and Japan. It was worthwhile testing different finenesses of lime in a UK field environment to reinforce to growers that lime fineness is an important consideration when selecting a lime treatment for control. The most effective control is achieved when using the finest liming materials with the majority passing through as small a sieve size as possible and preferably less than 150 µm (see Chapter 3). Hydrated lime, calcitic limestone and dolomite also work better the finer the particle size (Murakami *et al.,* 2002). The small

particles present alongside the granules of the calcium cyanamide product Perlka have also been shown to be essential for disease control (Donald *et al.,* 2004b).

Using finely divided lime applied in dry soil would be the best strategy for clubroot control because calcium would be uniformly incorporated into soil and would be readily available to plants even if sowing occurred soon after application. The uniformity of incorporation of lime is one of the most important factors in clubroot control (Dobson *et al.,* 1983).

The effect of adding shell sand on clubroot control in the field trials

The shell sand used in the field trials was made from crushed shells of whelk and scallop. The chemical analysis of this product showed that the 33% of its dry matter was calcium. It also contained high levels of aluminium. Other metals such as zinc, copper and manganese were present in the shells and they also contained a high level of sulphur.

Calcium is known to have a controlling effect against clubroot (Myers and Campbell, 1985) and the high content of calcium in this treatment might explain why the shell sand was effective at significantly reducing disease in the 2005 trial, but its effectiveness was not consistent (see results from 2006). Similar inconsistencies were observed for head weight and yield between trials. This could possibly be due to the fact that it was added at different times before transplanting in each trial (two weeks in advance of transplanting in the 2005 trial and only one day in advance in the 2006 trial). The effect on the soil pH and calcium in 2006 showed that shell sand increased soil pH within 24 hours of addition but thereafter fluctuated probably as a result of subsequent mobilisation of volatile compounds.

Shell sand also significantly increased the extractable calcium level within the first four days after it was added and then the level stayed stable for the rest of the trial. This is the same situation as with the lime treatments and may add more evidence to the

hypothesis that clubroot spores have to be in contact with high pH and high levels of calcium for a longer period than one day for them to be killed efficiently enough for disease to be reduced. When used in combination with the fungicide Shirlan, shell sand significantly reduced the disease level in the 2006 trial. This shows that not only could shell sand be used as a treatment on its own to control clubroot, it could be used in conjunction with other treatments to provide greater reductions in disease levels than could be achieved using single treatments. Shell sand is a waste product from the shellfish processing industry and would normally be thrown into landfill. Using this treatment could therefore not only be useful to brassica growers for controlling clubroot but could be a benefit to the shellfish industry by reducing the quantity that goes to landfill. Problems with using shell sand in farming include product inconsistencies in quality of this product as a result of natural variation.

The effect of Perlka on clubroot control in the field trials

Perlka, also known as calcium cyanamide, significantly reduced disease severity in both the 2005 and 2006 trials. It was significantly better at providing control than other treatments in the 2006 trial. Perlka had little effect on soil pH and calcium levels compared with the lime treatments; soil pH and calcium was maintained rather than increased. This contradicts the work of Murakami *et al.,* (2002) who reported that as more calcium cyanamide was added to the soil, the level of calcium in the soil and the pH of the soil increased. The effectiveness of Perlka at controlling clubroot was therefore not due to the effect on pH and calcium as is suspected with other calcium controlling compounds. Perlka degrades to hydrogen cyanamide when in contact with water. This compound possesses fungicidal properties (Murakami *et al.,* 2002). It is possible that this treatment killed spores, preventing the infection of the plants. This treatment has to be added to the soil in advance of transplanting because the high levels of nitrogen that this treatment initially releases into the soil can cause nitrogen phytotoxicity to the plants (Williamson and Dyce, 1989; Murakami *et al.,* 2002). It is recommended to add Perlka to the soil at least one day in advance for every 100 kg/ha of the product used (Paul Corfield, PP Products, pers. comm.). The addition of Perlka in

the 2006 trial took place one day before transplanting despite 500 kg/ha being added to the soil and yet the treatment significantly increased the head weights of the plant but not total yield. Some of the plants may have been killed due to suspected nitrogen toxicity, but the surviving plants had a higher head weight. This increase in head weight may also have been due in part to the increased nitrogen level in the soil caused by this treatment (Williamson and Dyce, 1989). In the 2006 trial, a second application of Perlka was applied to the soil 11 days after transplanting as recommended by Paul Corfield (pers. com.). This appeared to cause a slight increase in pH after day 11. This extra pH boost and increase in hydrogen cyanamide at the time when the main increase in auxin occurs in the plants for club formation (Ludwig-Muller *et al.,* 1996) could have worked in conjunction to lower disease severity by not only killing spores but by preventing the club formation on the roots. Perlka is considered to be an easy treatment to apply to soil because it is in a granular form and it is also used as a fertiliser. Growers consider the treatment expensive but by banding the treatments to triple the treatment rates as in this trial, it reduced the cost of the treatment and at the same time made it come into closer contact with the plant roots giving added protection to the roots.

Perlka was effective in both field trials despite the weather, soil nutrient conditions, spore race and disease pressure being different. This would suggest that Perlka could be a more consistent method for controlling clubroot than lime treatments as its effectiveness appeared to be not so affected by weather and other soil properties.

The effect of the fungicides Amistar, Ranman and Shirlan on clubroot control in the field trials

Shirlan (fluazinam) is active against oomycete fungi and *P. brassicae* because it appears to affect the respiration of resting spores (Humpherson-Jones, 1993). It had been used in field trials against clubroot in Australia and was shown to have controlling effects against disease especially when it was band incorporated into the soil as in the case of these two trials (Donald *et al.,* 2001). Ranman (cyazofamid) is a fungicide with high levels of activity against oomycete fungi and *P. brassicae* (Mitani *et al.,* 2003). Its

controlling effect against *P. brassicae* is due to inhibition of resting spore germination (Mitani *et al.,* 2003). Amistar is a strobulin based broad-spectrum fungicide that is registered for use in many crops including oilseed rape and is effective against *Sclerotinia sclerotiorum*, *Alternaria* spp. and *Botrytis* (Sudisha *et al.,* 2005). There is no published information with regards to its use against *P. brassicae.* All of these products are relatively new and each could potentially be an effective novel clubroot control measure.

The Ranman treatment significantly reduced the disease severity in the 2005 field trial. This treatment slightly, but not significantly, reduced head weight and yield compared with the controls and this is unlikely to have been due to phytotoxic action as there was no significant difference between the number of heads produced by this treatment compared with other treatments. This treatment was not re-tested in the 2006 trial as the UK industry was unlikely to see this product approved for use in brassicas because the manufacturer had no plans to support an application to the Pesticides Safety Directorate for approval for its use against clubroot.

Shirlan is also a protectant fungicide and is currently most commonly used to control foliar blight and tuber blight in potatoes. The aim was to determine if it would affect clubroot spores before they were able to enter the plants and prevent clubroot disease developing. However, Shirlan had no effect on disease severity in the 2005 trial and was the only treatment to significantly reduce the head weight and yield of the plants compared with the controls, indicating a phytotoxicity issue which was confirmed visually. Shirlan did not reduce disease severity as a single treatment in the 2006 trial either, but in combination with shell sand, it significantly reduced disease and therefore it might still have some merit as a control agent. Shirlan did not increase head weight or yield when it was added to the soil singly but when it was added in combination with calcium carbonate, significantly increased yield. Thus the phytotoxicity caused by Shirlan may be counterbalanced if other treatments are added alongside it.

Amistar is a fungicide that is effective against many types of fungi. However in these trials it showed no effect against clubroot because plots treated with this treatment had a DI of nearly 100. This result may indicate that other fungi in the soil could play a part in helping to control clubroot disease. Fungi such as *Heteroconium chaetospira* have been shown to control clubroot disease by occupying brassica roots, possibly preventing *P. brassicae* development within them due to a lack of physical space (Narisawa *et al.,* 2005). Therefore, killing many types of fungi in the soil may not be the optimal way of controlling clubroot. Also *P. brassicae* is a protozoan and therefore many fungicides of this type may not be effective against this organism. Amistar had no effect on plant head weight but it increased the plant yield showing that many plants survived but with smaller heads. The reason for this is unknown but potentially indicates that soil microflora may have a part to play in clubroot control and in plant growth.

The effect of the biocontrol agent Bactolife DP104 on clubroot control in the field trials

Bactolife DP104 is a commercially available biocontrol product which is based on an antagonistic strain of bacteria, *Bacillus subtilis.* It is a highly concentrated and complex culture of over 20 strains of microorganisms such as *B. subtilis, B. megateriam*, Rhizobia, Azobacteria and Saccharomyces (www.biotechnica.co.uk). It was hypothesised that these organisms would multiply in the soil and then control *P. brassicae* by producing anti-fungal compounds, cell-wall degrading enzymes, by inducing resistance of host tissues and by producing plant growth regulators. This treatment was only tested in the 2005 trial. It had no significant effect on disease or yield. Therefore, it was not tested in the soil in the 2006 trial. The rhizosphere is very complex and not well understood. Changing the balance of one type of organism could in turn affect other organisms that may be of benefit to the plants in ways that are still unknown. This treatment had shown some controlling effect against clubroot in glasshouse experiments (Chapter 3). Therefore, in the field, the level of Bactolife DP104 used may not have had a controlling effect on clubroot. This may have been because the product could have drained down the soil profile and might not have come into contact with spores. Also, this treatment was not added to the soil two weeks in

advance so there may not have been enough time for the microorganisms to multiply in the soil to high enough levels to have an effect. The soil microflora is known to have an effect on clubroot development (Narisawa *et al.,* 2005; Hjort *et al.,* 2007) but until it is clear as to what the mechanisms are behind this effect, adding biological control agents to the soil to control clubroot does not appear to be a useful management technique at present.

The effect of Quillaja on clubroot control in the field trials

Quillaja extract is a natural plant saponin that acts as a non-ionic surfactant. This product can reduce the surface tension of water and produce persistent foam. The saponin is made of cholesterol complexes which can emulsify lipids and therefore disrupt cell membranes (Baumann *et al.,* 2000). *P. brassicae* spores contain a high level of lipids (Knights, 1970; Moxham, 1983) and it was hypothesised that quillaja would have a controlling effect on clubroot by lysing spores. Quillaja can also activate cellular immune responses, reduce ammonia in the soil and can activate microbial growth (Osbourn, 1996). This treatment had no effect on disease index, head weight or yield of the plants. The rate that was used in this trial could have been too low and the treatment could have drained too quickly down the soil profile. This conclusion was reached because Quillaja was observed to have a controlling effect against disease in glasshouse experiments where the treatment would have reached high levels in pots due to the inability to drain out of the soil.

The effect of oilseed rape meal on clubroot control in the field trials

Rapeseed meal is a by-product of the crushing, expelling and extraction of oil from oilseed rape (*B. napus*). An experiment carried out at SAC Aberdeen showed that adding a low level of oilseed rape meal to soil could control clubroot disease (Elaine Booth pers. com). It was thought that the glucosinolates (mustard oils) in the rape meal may have biofumigant attributes and would therefore kill clubroot spores before they had a chance to germinate. Rapeseed meal has been shown to have activity against *Cylindrocladium parasiticum* an ascomycete fungus which causes black rot of peanuts

by infecting the roots of this plant immediately behind the root tip (Bhardwaj *et al.,* 1996) and *Aphanomyces euteiches*, an oomycete fungus that causes root rot of legumes (Pfender and Hagedorn, 1983). In the 2005 field trial, oilseed rape meal was added to the soil two weeks in advance of transplanting so that any biofumigant effect would have time to take effect in the soil. The oilseed rape meal was the only treatment tested in any trial that significantly increased the level of disease on the plants. It had no effect on head weight or yield. The reason for the increase in disease index when oilseed rape meal was added to the soil was unclear. A glasshouse experiment was therefore carried out to examine the reasons for this effect (Chapter 3). The results of the experiment showed that disease levels were increased the further in advance of transplanting that the rapeseed meal was added. Glucosinolates that are present in oilseed rape meal have been shown to increase the germination of clubroot spores. This would mean that spores exposed to this treatment may have been primed to infect the plants as soon as they were transplanted, and therefore could be expected to show greater development over time than spores not exposed to this treatment. This could be a reason as to why the clubs on plants exposed to this treatment were more developed than on control plants or plants exposed to any of the other treatments. Thus, oilseed rape meal could be used to promote either early germination of spores and death in the absence of an appropriate host, or early germination followed by another treatment to kill the zoospores directly. However, inconsistencies in the quality of the rapeseed meal, resulting from the different cultivars and growing conditions used may present difficulties in establishing a standard treatment that can give effective results.

The effect of adding rye as a companion plant on clubroot control in the field trials

Planting rye plants alongside the calbrese plants in the 2006 trial was carried out to see if the rye roots would act as a 'decoy' for the clubroot spores. Rye can be infected to the root hair stage of the *P. brassicae* life-cycle (Friberg *et al.,* 2005). Therefore, by adding the rye plants alongside the calabrese plants, some of the spores in the soil would be expected to infect the rye roots, taking some of the disease pressure away from the calabrese plants and therefore reducing the levels of disease on the brassica crop. Also,

because the spores that infected the rye roots would not develop into secondary zoospores, the overall build-up of new clubroot spores in the soil at harvest could be reduced. In the 2006 trial, the roots of the calabrese plants grown in conjunction with the rye plants were different to the roots of calabrese plants that had been grown on their own. Both root systems of the rye and calabrese appeared to combine and formed what looked like a root ball. The disease index of calabrese plants grown alongside rye plants was reduced slightly, but not significantly, and this may have been because the roots of the calabrese did not grow in the usual way and therefore *P. brassicae* could not complete its life-cycle as effectively as in plants where the roots were able to grow more normally. Adding rye as a companion plant with the calabrese gave the lowest yield results. This appeared to be because the rye plants were competing with the calabrese for space and nutrients and therefore the calabrese could not grow to its full potential. Plant competition would have to be taken into account when trying to develop this sort of companion planting treatment against clubroot in the field.

The effect of adding spent mushroom compost on clubroot control in the field trials

Spent mushroom compost had no significant effect on disease severity in either the 2005 or 2006 trial. Spent mushroom compost significantly increased the head weight and the yield of the plants in the 2006 trial but not in the 2005 trial showing that under certain conditions it can act as a fertiliser. Spent mushroom compost is normally made up of composted straw, hay, peat, horse manure, poultry manure and gypsum (Guo *et al.,* 2001). It was hypothesised that this treatment would contain chitinolytic organisms because mushrooms are made of chitin. *P. brassicae* spores also contain chitin and therefore chitinolytic organisms present in the SMC could potentially multiply in the soil and degrade the chitin component of the *P. brassicae* spores. The results would suggest that if this did occur, it was not to levels sufficient to offer significant and consistent disease control. Spent mushroom compost showed no effect on soil pH in the 2005 trial but an increase in the pH of the soil was observed within one day after incorporation, followed by a decline in the 2006 trial. This may have been due the fact that SMC contains lime which would increase both pH and calcium (shown in the chemical

analysis in Appendix 2). This was demonstrated because the spent mushroom compost gave an increase in soil calcium in the first two weeks after addition in the 2005 trial and in the 2006 trial, this increase was shown to have occurred on day one, after which the calcium levels stayed steady. Although this treatment did not have an effect on disease severity, it increased plant yield thereby mitigating the effect of clubroot on yield to some extent. It was also observed that adding it to the soil one day in advance of transplanting was better than adding it two weeks in advance in terms of increasing yield. SMC is often used to add nutrients to the soil and these nutrients could explain the increased plant yield in 2006. Spent mushroom compost is another waste product from industry. Inconsistencies in quality of SMC may create problems in the commercial use of SMC (assuming that a case can be made for its use) as well as explaining the differences observed between trial years.

Using combinations of treatments to control clubroot in the field

In the 2006 trial, treatments were added to the soil in combination to determine whether adding different compounds with different modes of action and acting at different times along the life-cycle could act in a synergistic way to control clubroot in the field. Combinations of limes, fungicides and biocontrol agents were used. Generally, adding treatments in combination did not give significantly better disease control than adding treatments to the soil individually. The only combination that significantly reduced disease was the combination of Shirlan and shell sand. Both treatments added to the soil on their own did not have a significant effect at reducing clubroot. A possible explanation for this result was that these treatments acted on different parts of the life-cycle. It could therefore have been possible that any spores that were not in contact with the Shirlan active compound fluazinam (which prevents resting spore germination (Humpherson-Jones, 1993)) could have germinated and were then controlled separately by the shell sand. Shell sand increases soil pH and calcium and therefore this treatment may have had a controlling effect on the zoospore and/or the stages of *P. brassicae* in the roots because high pH and calcium have been hypothesised to affect the development of the pathogen inside the roots (Webster and Dixon, 1991).

The combination of calcium carbonate and shell sand did not significantly reduce disease compared with the controls. These treatments may act on the same part of the *P. brassicae* life-cycle or in the same manner (by increasing soil pH and calcium) and therefore there may be no added benefit to disease reduction if they were added together.

It was also observed that the combination of Shirlan and calcium carbonate (which contains more calcium and raises the soil pH to a greater extent than shell sand) was slightly, but not significantly, less effective than the combination of Shirlan and shell sand at reducing disease. It has been shown that high levels of calcium and pH may degrade fungicides (Matthiessen and Warton, 2006). Therefore, adding calcium carbonate (providing high levels of calcium to the soil and raising pH to a high level) may be destructive towards the fungicide, whereas the shell sand (providing moderate levels of calcium and less of a rise in pH) would not degrade the fungicide to the same extent.

Adding combinations of treatments to the soil worked well in Australian field trials against clubroot (Donald *et al.,* 2001) and this may be due to the different soil properties, weather and microflora in Australian soil compared with Scottish soil. The field trials carried out in this study in Fife have shown that adding a treatment to the soil that adds a low level of calcium to the soil along with a fungicide may be an effective way to control clubroot in fields that have high levels of spores in their soil.

General comments on the differences and similarities between the results of the two field trials

More treatments were effective at controlling clubroot disease in the 2005 trial than in the 2006 trials. There may be many reasons for this. The greater disease pressure in 2006 may have overwhelmed some of the treatment effects. Other research has shown that treatments such as lime or nutritional amendments are not as effective at controlling clubroot when spore loads are high (Colhoun, 1961) and the ineffectiveness of limes in the 2006 trial at significantly reducing disease may therefore have been due to the

suspected greater spore load in the soil in this trial compared with the 2005 trial. The number of spores that infect a plant does not always correspond to the final disease severity seen (Jones *et al*., 1982). Therefore, the treatments tested in 2006 may have reduced spore inoculum in the soil or prevented the development of *P. brassicae* in the root to some extent, but a greater spore load could have resulted in a small number of spores surviving the effects of the treatments and progressing into the root cortex to cause clubbing symptoms.

Originally it was hypothesised that adding treatments to the soil two weeks in advance of transplanting would give time for the treatments to react in the soil and build up soil pH and calcium levels prior to transplantation. The 2006 trial however demonstrated that especially in the case of the fine powdered treatments, reactions could be rapid, reaching maximum pH and calcium levels in the space of one to four days after addition. Therefore, it may not be just the level of the pH and calcium achieved in the soil that acts as the control approach against clubroot, but the amount of time that the spores are exposed to this increase may also be important. Perhaps the increase in pH and calcium in the soil over a longer period of time affects the soil microflora (Buscott and Varma, 2005) and causes them to synthesise products that are beneficial to the plants at transplanting or that are detrimental to the *P. brassicae* spores.

In the 2005 trial there were large reductions in disease severity when the plants were exposed to the pH and calcium enhancing treatments, but this reduction in disease did not correspond with a significant increase in head weight of the plants compared with the control plants. This may be because the trial took place on raised beds which meant that water could easily drain down the soil profile. Stagnant pools of water found when raised beds are not used are conducive to clubroot development and can also reduce plant growth. This is because growing roots require high levels of oxygen and if they are in stagnant water they will become inactive and unable to take up nutrients (Khondaker and Ozawa, 2007). Therefore, the raised beds may have provided optimum conditions for plant growth so that they could overcome the detrimental effects of the

clubroot symptoms. In the 2006 trial which was also carried out on raised beds, SMC significantly increased the average head weight and yield of the calabrese compared with the control plots. This may show that this treatment was having a beneficial effect on plant growth despite the weather and soil conditions being optimum for clubroot development. This could suggest that if disease pressure is high in the soil, it may be beneficial to help increase plant yield and increase grower profits without necessarily reducing disease level. This theory could be tested by adding different spore loads to soil with increasing nutrients to determine whether head weight could be increased. If disease pressure in the soil is low, it might not be necessary to add treatments to the soil because raised beds alone may reduce the negative effect that clubroot has on plant yield.

How do these trials relate to other research on clubroot control?

The pH recommended for clubroot control is pH 7.2 or greater (Webster and Dixon, 1991). The pre-planting pH of the 2005 field trial was pH 7.5 and yet the control plots showed a high level of disease. Similarly, treatments such as calcium carbonate and calcium oxide used in the 2006 trial also raised the pH of the soil to above pH 7.2 and yet did not give significant control. Tremblay *et al.,* (2005) achieved good clubroot control in the field without reaching pH 7.2 whereas Campbell *et al.,* (1985) and Larson and Walker (1944) suggest that pH 7.2 is effective for clubroot control in the glasshouse but not in the field. This may be due to the properties of the different soil types used in the field experiments. Scottish soils are naturally acidic (Langan and Wilson, 1994) and therefore a treatment such as calcium oxide which can give higher pH increases in the soil compared with calcium carbonate may be beneficial in increasing the pH in Scottish soils to levels that are prohibitive towards clubroot development.

Campbell *et al.,* (1985) observed that a level of 2400 – 2800 mg/kg soil of extractable calcium would significantly reduce clubroot expression in a field environment. This was the level of the pre-planting soil calcium content in the 2005 trial and yet there was a high level of disease in the control plots in this trial. A higher level than 2800 mg/kg

soil of extractable calcium was also measured in several of the plots in the 2006 trials amended with treatments, however it did not always significantly reduce clubroot. Therefore, trying to control clubroot is not just about raising soil pH and calcium levels to a standard level as presented by other researchers (Campbell *et al.,* 1985).

In various experiments it has been shown that lime products that increase both calcium and pH are associated with the maximum reductions in symptoms compared with those that affect pH alone (Webster & Dixon, 1991). It has been argued that in the pH range 6.7 – 7.2, the balance between extractable cations in the soil and pH affects the degree of control achieved by liming (Campbell *et al.,* 1985). For example, at high pH, low cation concentrations are adequate to control clubbing. At low pH, high cation concentrations are needed to control clubbing (Webster & Dixon, 1991). This may relate to the observation that calcium uptake by plants is less at pH 6.2 than 7.2 with intermediate or low amounts of calcium in the soil (Myers & Campbell, 1985). Donald and Porter (2004) also showed that at a pH of 5.5, root hairs became rapidly infected and there was no effect from added calcium except at a high rate. At pH 6.5, all calcium amendments caused a significant delay in the development of *P. brassicae* but as the pH increased, the number of root hairs infected and the effect of calcium amendment decreased until at pH 8, 75-95% of root hairs were uninfected 10 days after inoculation and there was no significant effect of calcium on the development of *P. brassicae*. In the field however, high levels of exchangeable soil calcium did not relate to a high concentration of calcium in host roots (Donald *et al.,* 2006).

There is no straightforward answer as to whether it is pH or calcium that controls clubroot. Early researchers (Haenseler, 1939) advocated control using manipulation of pH whereas later researchers (Fletcher *et al.,* 1982) maintained that calcium ions alone could control *P. brassicae*. The interaction of cations and pH may be important for cation uptake and effect within the plant as well as for their affect on the organism (Campbell *et al.,* 1985). High pH and high levels of calcium may trigger similar mechanisms that inhibit the development of *P. brassicae,* or host and pathogen

responses to calcium may vary with pH value. High pH and high levels of calcium could also have a synergistic effect at controlling clubroot.

In *Paramecium*, an increase in calcium permeability in a response to depolarization of the membrane due to an increase in pH caused the flagellar motion of the organism to be altered (Naitoh *et al.,* 1972). In *P. brassicae* there may be a similar mechanism and the combination of high pH and calcium may be affecting zoospore motility.

Dixon and Webster (1988) showed that high pH and calcium together suppress root hair infection whereas high pH alone limits gall formation. Membrane permeability can be altered by high calcium and alkaline pH and this could affect intracellular phases of the parasite during proliferation. Protons can exchange with calcium ions at binding sites or cell membranes. This means that at an alkaline pH, more calcium could bind to cell membranes and enhance the inhibition of wall softening (Dixon and Webster, 1988). This could be why high pHs and high calcium together act synergistically against clubroot. High calcium also hyperpolarises cell membranes and activates H^+ATPases so that ion efflux down the electrochemical gradient is increased and intracellular auxin levels decrease (White, 1998). The effect could be pH dependent and would reduce cell hypertrophy which could lead to an inhibition of clubroot disease development. A depolarisation-activated Ca_2^+ current has been reported in protoplasts from root cells of *Arabidopsis thaliana*. Depolarisation-activated Ca_2^+ channels are thought to provide a generalised signalling mechanism indicating a breach in plasma membrane integrity and priming the cell for response (White, 1998). These channels may be involved in the defence response of brassicas towards *P. brassicae*.

Weather obviously played a part in the effectiveness of the treatments in controlling clubroot in these trials. This also confirms what other researchers have observed (Campbell *et al.,* 1985; Colhoun, 1953). Spore load, spore race, soil microflora, soil management, initial soil nutrients, pH and soil type all have to be taken into consideration when deciding what treatments to use for control.

These trials also demonstrated that the timing of the addition of treatments may be critical in achieving good clubroot control. Other research groups have come to the same conclusion (Campbell *et al.,* 1985; Murakami *et al.,* (2002). The trials also confirmed that fine powdered limes give better control than more coarsely ground types. This has also been shown by Dobson, (1983) and Murakami *et al.,* (2002).

Implication of these results on the improvement of methods to control clubroot

Perlka was the most effective treatment at controlling clubroot in both the 2005 and 2006 trial. It also significantly increased the head weight and yield of the plants in the 2006 trial compared with the control plants. This would suggest that Perlka is effective in controlling clubroot in trials with different soil and weather conditions. Comparing the results from the 2005 and 2006 trials, it could be speculated that Perlka has a greater benefit to plant yield if it is applied in two applications; one just before planting and the other 11 days later as secondary zoospores are forming. Adding this treatment in this way may be a good control strategy against clubroot and is different to current practice which recommends adding Perlka 7 – 10 days in advance of transplanting no matter what the rate used (Klasse, 1999).

The LimeX treatment worked well in the 2006 trial. In this trial, the disease pressure was very high and the weather conditions were optimal for clubroot development so it is likely that this treatment would have worked well in the 2005 trial when conditions were less optimal for clubroot development. LimeX gave the most significant disease control of the lime treatments tested in the 2006 trial and was the only lime treatment to give a significant increase in plant yield in either of the trials. Therefore, this lime treatment shows the greatest potential for practical use by growers in the UK. Novel treatments tested in these trials such as shell sand and SMC are variable in composition and may not provide uniform control each time that they are used but if there was further work determining how they act against *P. brassicae*, they could provide an alternative method of controlling clubroot to the conventional lime treatments. They may be of benefit for

farm land that is close to shell fish processing sites or mushroom farms as the carbon footprint for moving these substances would be low.

The results of the two field trials carried out in Fife have shown that pH and calcium in the field soil do have a part to play in clubroot control and that lime need not be added to the soil far in advance of planting to get a significant rise in soil pH and calcium as is normally recommended (Dobson *et al.,* 1983; Troeh and Thompson, 2005). Adding lime after harvest which is standard farming practice may not be useful in clubroot control because the earlier that limes are added to the soil, the more chance there is of the pH decreasing over time and of the leaching out of nutrients such as calcium so that the initial soil conditions at planting are favourable to clubroot. The field results from this study have shown that it may be better to add lime to the soil two weeks before transplanting to give the highest soil pH and calcium levels achieved by these treatments at planting. This high pH and calcium levels at planting may benefit the plant or make the soil conditions unfavourable for the infection of zoospores into the roots. It is a possibility that the LimeX treatment may have given even greater levels of control if added to the soil two weeks in advance like the limes tested in the 2005 trial. The 2006 trial also showed that combinations of treatments involving lime did not provide more significant disease reduction than if individual treatments were used singly.

The results of these trials are useful for other workers researching the development of clubroot control measures because they have indicated that compounds that contain calcium and increase soil pH other than conventional quarried lime may be effective in reducing disease levels. Fungicides that are effective specifically against oomycete fungi could also be a possibility as a new control measure. The trials also indicated that the length of time that spores are exposed to high pH and calcium levels, or that high levels at planting may play a role in disease development. Therefore, the timing of addition of different lime treatments in field soil to achieve the optimum levels at transplanting could be researched.

The trials have shown that using raised beds and applying treatments in bands to concentrate the treatments around the roots could be a useful way of increasing the treatment rate without increasing the expense and enhancing the chance of treatments coming into contact with the spores when they enter the root zone.

The trials have also indicated that weather conditions, spore load, nutrient level, soil glucosinolate content and soil microflora (as seen by the Amistar results in 2006) may have a part to play in the efficacy of treatments for control and further research into the mechanisms of how each factor affects disease and how they can all interact to reduce the effectiveness of clubroot control treatments could be useful in developing new control measures.

Further work needed

In future field trials examining the effectiveness of treatments against clubroot, more treated plots could be sampled at more time points for pH and calcium levels. This may show when the highest pH and calcium levels are achieved in the soil after addition of the treatments, and may provide information on how far in advance the treatments should be applied before transplanting to give high pH and calcium levels in the soil at planting. This would be time consuming and expensive but may be important in optimising the addition of conventional treatments to achieve consistent clubroot control.

The spore load in each of the treated plots could be determined throughout the duration of the trial. This would determine whether the treatments were having an effect on the spores in the soil and were preventing disease by reducing spore level, or if they were affecting the plant directly. The development of a quantitative real-time polymerase chain reaction (PCR) test that could measure the level of viable *P. brassicae* spores directly from soil would be useful for this purpose.

Different rates and combinations of conventional and novel treatments could be tested in future trials. Testing combinations requires large trials which may be prohibitive in researching different combinations of many treatments. Treatments that act on different life-cycle stages may be hypothesised to have the greatest effect on clubroot control if added to the soil together, so determining where each treatment operates in the life-cycle to control clubroot is important in considering which treatments to test in combination.

Field trials where the same treatments are added at the same rate and time before transplanting to different soils in different years should be carried out. This would allow a direct comparison of treatment effectiveness in different soils to be made. Trials on the same location over a number of years would also be useful to determine the ability of various treatments to control clubroot in a specific soil type consistently over a number of years.

Examining the correlation between individual plant disease scores and head weights would also provide more insight into the effect of the treatments on disease, plant growth and yield. Vigour and toxicity data should also be recorded to dismiss any confounding effects on disease control and yield because of phytotoxicity.

Glasshouse trials examining the effect of soil microflora on clubroot and plant growth would greatly enhance the knowledge of the disease under natural conditions and could potentially help in the development of control measures. Organisms that were found to specifically suppress clubroot development could be added to the soil, and organisms that were found to promote clubroot could themselves be targeted with control treatments to enhance disease control. Microorganisms that are found naturally in British soil could be added to pots and altered in level and combination to examine what effect, if any, they have on clubroot. This knowledge could be used to examine why treatments used to control clubroot are not always effective in the field environment.

Conclusions

The two field trials carried out in Fife in this study have shown that clubroot control is variable and cannot be controlled consistently using the same treatments at the same rates in different fields under different conditions. The pH and nutrient status of the soil, spore loading and race, timing of the addition of treatments and the weather conditions can all influence the effectiveness of treatments and were all hypothesised to affect the effectiveness of the treatments to control clubroot in these fields. Perlka at the rates and timings of application used in these trials gave significant control of clubroot in the field even under optimal conditions for clubroot development. There was evidence that where lime was used to control clubroot in the field, fine powdered forms were more effective and best applied around two weeks before transplanting to give an increase in pH and calcium in the soil by planting. However, LimeX was also shown to be a promising treatment for clubroot control even when added to the soil immediately before transplanting. Novel treatments such as shell sand and spent mushroom compost had limited effect on reducing disease levels and inconsistencies in quality of these products could also rule them out of being used as novel clubroot control measures. Combinations of treatments such as a fungicide that specifically acts against oomycete fungi (e.g Shirlan) combined with a treatment that provides a moderate amount of calcium to the soil may also help to provide more effective control than single treatments if these combinations and rates could be optimised, however, it was generally shown that no significant extra benefit in disease control was achieved by combining treatments. The key to clubroot control in the field is complex and more work has to be done on repeating these experiments in different types of soil with different spore loads and different weather conditions to develop treatments that give consistent control across varied situations.

CHAPTER 5 – DEVELOPMENT OF A REAL-TIME PCR QUANTITATIVE DIAGNOSTIC TEST FOR THE PRESENCE OF PLASMODIOPHORA BRASSICAE RESTING SPORES DIRECTLY FROM SOIL

Introduction

The control of clubroot disease of brassicas using treatments such as lime, fungicides and calcium cyanamide can be inconsistent and expensive. Part of the reason for the inconsistencies in control is thought to be because of variations in the initial spore load in the soil. Researchers have shown that in soils where spore loads are high, liming will not be effective at preventing disease (Hamilton and Crete, 1978). Also, resistant cultivars of spring oilseed rape have been developed that show no disease when the spore load in the soil is low, but are not fully resistant if the spore loads are high (Wallenhammar and Ardwisson, 2001). Therefore, it would be useful for brassica growers to determine the initial spore load in their field soil so that appropriate control measures can be carried out or fields with very high spore loads can be avoided.

Currently, a bait plant test is the most common method used to test for the presence of *P. brassicae* spores in field soil. This bait test involves planting highly susceptible Chinese cabbage seedlings into a sample of field soil and assessing clubroot disease development on the roots. This method can detect inoculum levels of greater than 1000 spores/g dry soil (Wallenhammar, 1996) but can only indicate whether disease severity would be moderate or high under glasshouse conditions. This method is resource heavy, it takes a minimum of six weeks to carry out, the results can be affected by environmental conditions and it requires a specialist to check for disease development on the roots which puts high demands on staff time. Using fluorescence microscopy to detect clubroot spores in soil also requires a specialist who is trained to differentiate the spores from other fluorescent objects in the soil. The binding of spores to soil particles can also result in an under-estimation of the spore load using this technique (Faggian *et al.,* 1999). This method is therefore not very practical for the development of a rapid, accurate diagnostic test. Serological detection is also not an effective method for estimating the numbers of resting spores in the soil. This is because there are many races of *P. brassicae* and because the sample preparation is very laborious (Faggian *et al.,* 1999). A test to detect *P. brassicae* spores using monoclonal antibody technology on a lateral flow test device is currently being developed (Donald *et al.,* 2006). This

kind of test has the potential to be made quantitative and could be used on farm by the grower, but currently, the biggest challenge in developing this kind of test is developing a reliable way to extract the pathogen from the soil (Donald *et al.,* 2006).

A rapid, sensitive and specific test for detecting and quantifying the numbers of *P. brassicae* resting spores in the soil is therefore still required. The detection of the presence of *P. brassicae* DNA in the soil using PCR (polymerase chain reaction) would reduce the need for a specialist to analyse plant roots for disease development or detect spores under a microscope. Using specific primers for the organism would reduce the chance of cross-reactivity in a test which is sometimes observed in serological tests (Donald *et al.,* 2006). The test could also be carried out in laboratory conditions by a specialist who would be able to relate the results to a field environment.

The use of real-time PCR would be an ideal method to use for the development of this test. This is because real-time PCR allows the starting level of DNA present in a sample to be quantified, and so theoretically, the initial spore load in a soil could be accurately measured using this technique. Primers and probes that are specific to *P. brassicae* have already been developed and so the main challenge in developing a real-time PCR diagnostic test for the quantification of *P. brassicae* resting spores in the soil was to extract the spores directly from the soil. This would decrease the amount of time taken to carry out a diagnostic test because a plant host step would not be needed. Another aim was to make the test quantitative by creating standard curves of certain spore loads in the soil, and relate the results to the whole field environment.

Extracting DNA from soil and amplifying the DNA of one selected microorganism can be challenging. Soil contains a very wide range of microbial genetic diversity so the primers have to be specific to *P. brassicae*. DNA extraction from soil is also hindered by the complexity of soil properties such as varying soil texture and structure, water content, pH and organic matter. All of these things can hinder the extraction of DNA

from soil, and substances like humic acids can inhibit the PCR reaction (Cankar *et al.,* 2006).

The aim of the experiments carried out in this study was to extract *P. brassicae* DNA from soil using selected extraction methods and amplify the extracted DNA using sets of primers designed specifically for *P. brassicae* to show that *P. brassicae* could be successfully detected directly from soil DNA extractions. To make the test quantitative, a set of standard *P. brassicae* DNA levels that could be compared with the DNA extracted from naturally infested field soils had to be developed which could give an estimation of the number of spores present in the soil. The problems encountered when developing a quantitative PCR-based test for a soil-borne obligate pathogen like *P. brassicae* is discussed.

When developing a quantitative PCR test for an obligate soil-borne pathogen like *P. brassicae*, many different factors have to be taken into account. Successful quantification depends crucially on the quality of the sample DNA analysed (Cankar *et al.,* 2006). The quality of DNA concerns not only the purity but also the structural integrity of DNA obtained and the term PCR forming units (PFU) was introduced to distinguish between structurally intact and damaged (non-amplifiable) target DNA copies (Cankar *et al.,* 2006). Therefore another major aim of the study was to discover inexpensive methods of soil DNA extraction that could be used routinely to extract high quality DNA from soil.

Materials and methods

Plant and soil sources for DNA extraction

P. brassicae infected Chinese cabbage roots

Six week old Chinese cabbage cv. SB1 Kilo plants that were infected with *P. brassicae* had been prepared using the slurry method (see Chapter 2). The spore population was determined as 16/31/31 using the European Clubroot Differential (ECD) method (see

Chapter 2). The galled roots had been washed free of soil and were put in plastic bags and stored at -20^{o}C until required.

Sources of naturally infested field soil

The field soil samples used throughout these experiments were taken from naturally infested fields throughout the UK. The 'Aberdeen' soil came from the 'P & Q' beds of the SAC Aberdeen Craibstone estate (see Chapter 2). The 'Fife' soil came from Kirkmay farm, Crail, Fife (see Chapter 5). The 'Lincolnshire' soil came from field T2 of J & J Tunnard Ltd., The Chestnuts, Green Lane, Algakirk, Boston, Lincs. A 5 kg soil sample was taken from each site by taking random samples of soil from throughout the field to a depth of 10 cm and mixing them together. Two field soils from Fife, one that had been shown by a bait plant test to give a high level of clubroot disease in brassicas, and another that showed no clubroot disease in a bait plant test were also used in the real-time PCR assays.

Inoculation of John Innes compost with *P. brassicae* spores

John Innes No. 1 compost (pH 5.5) with selected amounts of spores added to it was used as positive control soil throughout the experiments. Spore suspensions were made using the method seen in the ECD chapter for the preparation of inoculum. To inoculate the soil, 500 g of John Innes No. 1 compost was put into a plastic bag. A 50 ml spore suspension containing a specific amount of spores between 10^4 to 10^8 spores/ g ml (determined using a haemocytometer) was then poured onto the soil and the plastic bag was shaken and the soil was mixed so that all of the soil was wetted with the spore suspension.

DNA Extractions

Extraction of *P. brassicae* DNA from infected roots of Chinese cabbage

DNA was extracted from galled roots of Chinese cabbage using eight different DNA extraction methods (see Table 5.1). The leaf disc protocol of the REDExtract-N-Amp Plant PCR kit from Sigma was used on the roots. A 2 mm diameter section of galled

Chinese cabbage root was used instead of leaf tissue. The extraction method normally used for Drosophila was used with the Puregene DNA isolation kit from Gentra Systems, replacing Drosophila tissue with a 2 mm root section. The plant standard protocol 1 of the Nucleospin kit from Machery-Nagel was tested. 100 mg of frozen infected root was used and the root was homogenised using a craft drill with a conical head attachment (Minicraft Variable Speed Drill Kit, MB 150, 12v) for 10 seconds. The optional addition of 10 µl of RNase A solution in this standard protocol was carried out. The standard protocol of the DNeasy plant mini kit from Qiagen was also tested. 70 mg of frozen infected root was used and the root was homogenised using a minicraft drill for 10 seconds. The optional centrifugation of the lysate of their standard method was carried out. A method described by Wallenhammer *et al.,* (2001) was used as a way to extract DNA from the roots without the use of an expensive kit. Two cm long sections of infected roots were used. 200 µl of 1M Tris-HCl, pH 4.8 was added to the roots in a 1.5 ml Eppendorf tube. The roots were crushed using a microfuge tube pestle. The tube was placed in a heat block at 100^{o}C for 10 minutes to damage the tissue and extract the DNA. The tube was quickly cooled in ice. The liquid was centrifuged at 2500 x g for 8 minutes to pellet root material. The supernatant was removed and this contained the extracted DNA. An altered version of the Wallenhammer method was also tested to determine the effect of a different pH of Tris-HCl buffer on level and quality of DNA extracted compared to the initial method. In this method, the sample was incubated at 100 ^{o}C for 15 minutes instead of 10. The sample was homogenised quickly (10 seconds) using a craft drill with a conical head attachment. The pH of the Tris-HCL solution was altered to pH 7.5 using NaOH.

A Fast Prep FP120 machine from Bio 101 systems was used to extract DNA from clubbed tissue. This machine homogonises tissue mechanically by shaking tissue in tubes at a vertical angular motion in a lysing matrix of sand and beads for optimal DNA extraction with reduced nucleic acid shearing. The Fast Prep DNA extraction kit from Bio 101 systems was used with this machine. The ¼ sphere and garnet mix was used as the lysing matrix in the tubes to shear the root tissue. The machine speed was 4.5 for 30

seconds and the amount of clubbed root tissue used was 0.15 g. Another extraction method using this machine was tested by adding 1 ml of Tris-HCl pH 7.5 to the lysing matrix tubes with 0.15 g of root tissue in them and then putting them in the Fast Prep machine at a speed of 4.5 for 30 seconds. This was to determine if the tissue homogenising machine could be used to extract DNA without using the kit that was supplied with the machine in an attempt to reduce costs. The shaken tubes were then put into a heat block at 100^{o}C for 10 min and were then quickly cooled in ice. The tubes were centrifuged at 2500 x g for 4 minutes. The supernatant was saved to provide the DNA sample. DNA extractions were replicated three times using each extraction method.

Table 5.1. DNA extraction methods used for extracting DNA from *P. brassicae* infected roots of Chinese cabbage

DNA extraction method	Supplier	Time taken for DNA extraction	Method of extraction
Boiling method (Tris-HCl pH 4.8)		30 minutes	Heat shocking tissue to extract DNA
Boiling method (Tris-HCl pH 7.5)		30 minutes	Heat shocking tissue to extract DNA
DNeasy	Qiagen, UK	45 minutes	Mechanical disruption and lysis of tissue using enzymes
Fast Prep kit	Bio 101 systems, Cambridge, UK	40 minutes	Energetic mechanical disruption of tissue reducing the need for lysis enzymes
Fast Prep Tris-HCl pH 7.5	N/A	40 minutes	Energetic mechanical disruption of tissue followed by heat shocking
NucleoSpin	Macherey-Nagel, Cambridge, UK	50 minutes	Mechanical disruption and enzymatic digestion of tissue
Puregene	Gentra systems, UK	2 hours 30 minutes	Enzymatic digestion of tissue
RED-Extract N-Amp Plant PCR kit	Sigma-Aldrich, UK	15 minutes	Enzymatic digestion of tissue

Extraction of *P. brassicae* DNA from infected soil using commercial kits

Two kits were evaluated for their efficacy at extracting DNA from field soil infested with *P. brassicae* resting spores and soil that had been artificially inoculated with resting spores. DNA was extracted from the three naturally infested field soils (Aberdeen, Fife and Lincolnshire) and John Innes compost inoculated with 10^5 spores/g soil using the PowerSoil DNA Isolation kit from Mo Bio. The standard protocol was followed. 250 mg of each soil was used in the extraction process. This 250 mg of soil was randomly sub-sampled from the larger 5 kg samples of soil taken from the naturally infested field soils and from the 500 g of artificially inoculated compost.

The PowerMax Soil DNA Isolation Kit from Mo Bio was also used to extract DNA from the three naturally infested field soils and John Innes compost inoculated with 10^5 spores/g soil. The standard protocol was used. With this kit, 10 g of soil sub-sampled randomly from the initial soil samples were used. DNA was only extracted once from each soil using the commercial soil DNA extraction kits.

Retsch mill soil DNA extraction

A piece of machinery called a Retsch planetary ball mill (Retsch Gmbh, Haan, Germany) was used to help pulverise soil into a very fine powder so that DNA could be extracted from it. This machine can crush 60 g samples of soil. A method of DNA extraction using this machine was developed by the Scottish Crop Research Institute (SCRI) in Dundee and was followed in these experiments.

Firstly, a 60 g sub-sample of each soil sample (naturally infested field soil or John Innes No. 1 compost that had been inoculated with a dilution series of spores) were put into plastic bags and any lumps present in the soil were flattened with a roller. The bags were inflated and the soil was shaken in the bags to mix it. Each 60 g soil sample was then added to one of the grinding jars of the Retsch mill, and 120 ml of CTAB-PO_4 buffer and 12 steel balls were added to the jar. The machine was then used to crush the soil following the standard protocol of the Retsch mill operation booklet.

Four lots of 1.5 ml aliquots were taken from each jar and were put into 2 ml Eppendorf tubes. Another 20 ml of each soil slurry was frozen in 50 ml tubes in case it was needed for future analysis. The 1.5 ml aliquots of soil slurry were centrifuged at 6000 x g for 5 minutes. The supernatant was removed and put into new 1.5 ml centrifuge tubes where 0.9 ml of cold chloroform was added to it. The samples were vortexed and placed in a microcentrifuge tube at 13 000 x g for 4 minutes. 900 µl of aqueous phase from this step was put into new tubes. 90 µl of 3M sodium acetate was added to the tubes and the tubes were vortexed and left at room temperature for 1 hour. The tubes were then spun at 13 000 x g for 4 minutes after which the liquid was removed and discarded. The remaining pellet was washed by adding 150 µl of 70% ethanol to the tubes and then spinning the tubes at 13 000 x g for 2 minutes. The ethanol was then removed and the pellet was left to air dry for 10 minutes. The pellet was then re-suspended in 100 µl 1 x TE buffer and was vortexted to break up the pellet. The tubes were left overnight in a 4^{o}C fridge to help the pellet dissolve.

The next stage was to extract humic acids (which may have inhibited the PCR process) from the extracted DNA. Bio-Rad Micro Bio-Spin Chromatography columns were filled with polyvinylpolypyrrolidone (PVPP) powder to a height of 15 mm. The bottoms were then snapped off these columns and the columns were placed into individual 1.5 ml Eppendorf tubes. 150 µl of HPLC water was added to each column and the tubes were spun at 5000 x g for 3 minutes. This last step was repeated and then each column was placed in a new labeled 1.5 ml Eppendorf tube and the surface of the PVPP powder was roughed up with a pipette tip to stop any DNA from going down the sides of the powder. The 100 µl of DNA suspended in TE buffer was then added to the top of the column and the tubes were centrifuged at 5000 x g for 3 minutes to elute the DNA.

Quantification and storage of extracted DNA

The level of double stranded DNA extracted using each method was measured spectrophotometrically using a dilution of 60 µl of sterile de-ionised water and 2 µl of extracted DNA sample. All DNA samples were stored at -20 ^{o}C until needed.

Visualisation of extracted DNA and PCR products

Five µl of the extracted DNA or amplification product from the PCR reactions was added to 0.5 µl of MegaFluor dye and 0.5 µl MegaFluor loading buffer (Euroclone) and were analysed on a 1.5% agarose gel, visualised under UV light.

PCR amplification

Nested PCR

PCR amplification of infected root DNA extracts

A nested PCR method for the detection of *P. brassicae* DNA was based on four primers PbITS-1, PbITS-2, PbITS-6 and PbITS-7 designed by Faggian *et al.,* (1999) from the ITS1, 5.8S and part of the 18S and ITS2 regions. All primers were supplied by TAG Newcastle Ltd. and are described in Table 5.2. All amplifications were performed on an Applied Biosystems GeneAmp PCR system 9700 with the following reaction mix: 1 X GeneAmp PCR buffer II (10 mM Tris-HCl pH 8.3, 50mM KCl) from Applied Biosystems, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1% formamide, 1 µM of each primer, 20 µl of 0.5ng /µl root DNA extract (DNA was diluted to this level using sterile de-ionised water) and 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a total reaction volume of 50 µl. The reaction mixture was made up to 50 µl using sterile de-ionised water. In the nested PCR step, 1µl of the first amplification was used as a template for the second PCR. PCR conditions were 10 min at 94^{o}C, 45 cycles of 1 min at 94^{o}C, 1 min at 60^{o}C, 2 min at 72^{o}C and a final extension of 5 min at 72^{o}C following methods developed by Wallenhammar and Ardwisson (2001).

Another nested PCR method for detecting *P. brassicae* DNA was based on the outer primers of PBAW 10 and PBAW 11, or PBAW 12 and PBAW 13 designed by Wallenhammer and Ardwisson (2001) and the nested primers PBTZS 3 and PBTZS 4 designed by Ito *et al.,* (1997) from a suspected isopentyltransferase gene. All primers were supplied by TAG Newcastle Ltd, and are described in table 5.2. All amplifications were performed on an Applied Biosystems GeneAmp PCR system 9700 with the

following reaction mix: 1 X GeneAmp PCR buffer II (10 mM Tris-HCl pH 8.3, 50mM KCl) from Applied Biosystems, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 µM of each primer, 20 µl of 0.5ng /µl root DNA extract and 1.75 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a total reaction volume of 50 µl. In the nested PCR step, 1µl of the first amplification was used as a template for the second PCR. PCR conditions were 3 min at 94°C, 40 cycles of 1 min at 94°C, 2 min at 50°C, 1 min at 72°C and a final extension of 5 min at 72°C following the methods developed by Ian Barker, (pers. com).

Optimisation of PCR annealing temperature

A thermal cycler machine (Thermo Hybrid MBS 0.2G) was programmed with an annealing temperature gradient to try and optimise the annealing temperature used in the PCR reaction when the Faggian *et al.,* (1999) designed nested primers were used. The gradient was set up at the 60°C step of the cycling conditions. The annealing temperatures tested were 62.8°C, 64.5°C, 66.4°C, 68.4°C, 70.4°C and 72.0°C.

Table 5.2. Nuleotide sequence of primers used in nested PCR reactions

Primer (outer or nested)	Sequence (5'-3')	Reference
PbITS-1 (outer)	ACTTGCATCGATTACGTCCC	Faggian *et al.,* (1999)
PbITS-2 (outer)	GGCATTCTCGAGGGTATCAA	Faggian *et al.,* (1999)
PbITS-6 (nested)	CAACGAGTCAGCTTGAATGC	Faggian *et al.,* (1999)
PbITS-7(nested)	TGTTTCGGCTAGGATGGTTC	Faggian *et al.,* (1999)
PBAW 10 (outer)	CCCCGGGGATCACGATAAATAACA	Wallenhammar and Ardwisson (2001)
PBAW 11 (outer)	GGAAGGCCGCCCAGGACTACC	Wallenhammar and Ardwisson (2001)
PBAW 12 (outer)	GCCGGCCAGCATCTCCAT	Wallenhammar and Ardwisson (2001)
PBAW 13 (outer)	CCCCAGGGTTCACAGCGTTCAA	Wallenhammar and Ardwisson (2001)
PBTZS 3 (nested)	CCACGTCAGATCACGTTGCAAT	Ito *et al.,* (1997)
PBTZS 4 (nested)	CCTGGCGTTGATGTACTGGAA	Ito *et al.,* (1997)

Real-time PCR

During real-time PCR unlike conventional PCR, the accumulation of PCR products is measured automatically after each cycle using an integrated cycler/fluorimeter. The initial amount of target DNA in the reaction can be related to a 'cycle threshold' (C_t), defined as that cycle number at which a statistically significant increase in fluorescence is detected. Target DNA can then be quantified by constructing a calibration curve that relates C_t to known amounts of template DNA (McCartney *et al.,* 2003). PCR products can be monitored using either fluorescent DNA-intercalating dyes (e.g. SYBR Green) or sequence-specific probe-based assays.

A real-time PCR reaction was carried out using the primers PBTZS 3 and PBTZS 4 (Table 5.2) using the DNA interchelating fluorescent dye SYBR Green (Qiagen) to determine if these primers could be used to detect *P. brassicae* DNA using a real-time method. Real-time PCR amplifications were performed on a BioRad iCycler real-time PCR machine in 96-well plates. Real-time PCR mixtures contained 10 µl of iQ SYBR Green Supermix (Qiagen), 1 µM of each primer, 10 µl of DNA template containing 1 ng DNA/µl and sterile de-ionised water to make the reaction volume up to 25 µl. The real-time assay temperature cycles were 3 min at 95^{o}C, and 45 cycles of 30 seconds at 94^{o}C, 30 seconds at 60^{o}C, 30 seconds at 72^{o}C, 30 seconds at 95^{o}C, 30 seconds at 50^{o}C and 10 seconds at 50^{o}C following the method of Faggian *et al.,* (2003).

Sets of primers and probes that had separately been developed for *P. brassicae* from the ITS1, 5.8S and part of the 18S and ITS2 regions by Central Science Laboratory (CSL), York (Ian Barker, pers. com.) and Faggian *et al.,* (2003) were also tested for their ability to detect *P. brassicae* DNA using real-time PCR. These primers can be seen in Table 5.3. The probes were labeled with the fluorescent dyes 5-carboxyfluorescein (FAM) on the 5' end and N,N,N,N,-tetramethyl-6-carboxrhodamine (TAMRA) on the 3' end. When the dyes are close together, the 5' reporter fluorophore (FAM) transfers laser-induced excitation energy to the 3' quencher (TAMRA). When the oligoprobe hybridises to its template, the fluorophores are released by the 5'-3' exonuclease activity

of the *Taq* polymerase, leading to the separation of the dyes in solution. Once they are separated, the reporter emission is no longer quenched and it is possible to measure the increasing fluorescence which is proportional to the amount of target DNA produced (Rondini *et al.,* 2003).

Real-time PCR amplifications were performed on a BioRad iCycler real-time PCR machine in 96-well plates. Real-time PCR mixtures contained 0.5 X GeneAmp PCR buffer II (10 mM Tris-HCl pH 8.3, 50mM KCl) from Applied Biosystems, 2.75 mM $MgCl_2$, 250 µM of each dNTP, 7.5 pmol of each primer, 5 pmol of probe, 10 µl of 1 ng /µl DNA extract (DNA was diluted to this level using sterile de-ionised water) and 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems) in a total reaction volume of 25 µl. The real-time assay temperature cycles were 2 min at 50°C, 10 minutes at 95°C, and 40 cycles of 2 min at 60°C and 15 seconds at 95°C following the method developed by CSL York (Ian Barker, pers. com.).

All real-time PCR analyses were done in triplicates. Negative controls containing sterile de-ionised water instead of DNA were included in each amplification experiment.

Table 5.3. Nucleotide sequence of primers and probes used in the real-time PCR reactions

Primer or probe	Sequence (5'-3')	Reference
CSL forward	CCCATATCCAACCCCATGTG	Ian Barker, Personal communication
CSL reverse	GAAAATGAAACACGCAGCTGG	Ian Barker, Personal communication
CSL probe	FAM-ACCGGTGACGTGCGGCGAC-TAMRA	Ian Barker, Personal communication
Aus forward	CGCTGCATCCCATATCCAA	Faggian *et al.,* (2003)
Aus reverse	TCGGCTAGGATGGTTCGAAA	Faggian *et al.,* (2003)
Aus probe	FAM-CGCACGTCACCGGTTCACAGG-TAMRA	Faggian *et al.,* (2003)

Standard curve

A standard curve was developed for the level of *P. brassicae* DNA extracted from soil by plotting the logarithm of known concentrations of spores extracted from soil (John Innes no. I compost that had a ten-fold dilution series of spores added to it and the DNA extracted using the Retsch mill method) against the cycle threshold (C_t) value. The C_t value is inversely proportional to the log of the initial concentration, so that the lower the C_t value, the higher the initial DNA concentration. The reason for plotting the DNA level against the C_t value was to determine the cycle number at which a statistically significant increase in fluorescence was detected at each spore load. DNA from unknown field samples could then be quantified by constructing a calibration curve that related C_t to known amounts of spores that had been extracted from the soil.

Analysis of DNA band in negative controls using Faggian *et al.*, (1999) designed nested primers

A band of DNA was always present after the second round of nested PCR in the negative control samples when using the Faggian *et al.,* (1999) designed nested primers. This band was observed even when methods such as using new master mix containing PCR components which had never been exposed to *P. brassicae* DNA were used. Therefore, attempts were made to sequence this DNA band to examine whether it was caused by *P. brassicae* DNA contamination of the negative controls or if there was another possible source of contamination.

Cloning the DNA fragment for sequencing using the TOPO TA Cloning kit from Invitrogen

The 'One Shot Chemical Transformation' protocol included with the TOPO TA Cloning kit (Invitrogen) was used to clone the DNA band from the negative controls into *E. coli* for future sequencing. To set up the TOPO cloning reaction, a 6 µl TOPO cloning reaction was made for transformation into chemically competent *E.coli* (Table 5.4).

Table 5.4. Reaction mixture for the TOPO cloning reaction

Reagent	Quantity (µl)
Fresh PCR product	4 µl
Salt solution	1 µl
TOPO Vector	1 µl

The fresh PCR product was the nested PCR product from the control tube. The transformed *E.coli* were spread onto a pre-warmed selective plate (Liquid Broth + 100 µg /ml ampicillin) and incubated at 37°C for 20 h. Twenty individual transformed colonies were picked off the plates using a sterile 10 µl pipette tip. They were streaked out onto a new liquid broth plate containing 100 µg /ml ampicillin. The plate was incubated at 25 °C for 48 h to achieve good bacterial growth.

PCR of cloned fragments

The transformed colonies of *E.coil* had their DNA amplified using PCR in order to have enough product for sequencing. A PCR master mix was assembled as in Table 5.5. A total of 20 transformed colonies and one negative control containing no DNA were used. Using a 10 µl pipette tip, a scraping of cloned fragment from each of the 20 transformed colonies was put into each PCR tube.

Table 5.5. Components of PCR master mix used to amplify the genome of transformed *E.coli*

Component	Volume per reaction (µl)
1.2 Units of Promega Taq	0.24
Promega Taq Buffer	5
2mM dNTPs	5
M13 forward primer	0.5
M13 reverse primer	0.5
Sterile de-ionised water	38.6
Total volume	50

PCR was carried out on the transformed *E. coli* DNA on a Biometra PCR machine using the following standard conditions: 5 min at 94°C, 5 min at 55 °C, then 35 cycles of 1.5 min at 72°C, 45 seconds at 94 °C and 50 seconds at 55 °C followed by 10 minutes at 72°C.

Extraction and purification of plasmid DNA

A streak of one of the transformed colonies was put into a 50 ml tube containing 10 ml of liquid broth and ampicillin. It was left shaking for 18 h at 37 ^{o}C. The transformed plasmid was extracted from the TOPO cells using the High Pure Plasmid isolation Kit from Roche. The conformation of plasmid DNA being present was shown on gel electrophoresis on a 0.8 % agarose gel run at 100 v for 0.8 hours.

Restriction digest of plasmid

A restriction enzyme digest of the extracted plasmid DNA with EcoR1 was carried out to ensure that the DNA that was sent to be sequenced contained the cloned DNA fragment from the original negative control sample. The correct sized insert was present – visualised by running the restriction enzyme digest on a 0.8% agarose gel run at 100 v for 0.8 hours. The sample was sent to DNASHEF technologies, Edinburgh to be sequenced.

Comparison of negative control DNA sample with known DNA sequences

The 507 bp insert was matched with *P. brassicae* DNA using the nucleotide BLASTn program on the website http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi.

Results

Commercial kits and various alterations of a boiling method were used to extract DNA from *P. brassicae* infected Chinese cabbage roots. High quality and a high yield of DNA would be needed to provide DNA for use in downstream PCR applications. The effectiveness of each method at providing these requirements and also the time taken to carry out each DNA extraction can be seen in table 5.6. The commercial kits gave a better quality of DNA than the methods that involved boiling the roots in Tris-HCl. The quality of the DNA was determined by visualising the DNA on an agarose gel. A single band of DNA was determined to be of high quality because if the DNA was spread over the whole gel it would demonstrate that the DNA had been degraded into small fragments. The Tris-HCl methods generally gave the highest yields of DNA out of the

methods tested. The DNA extracted would be a mixture of plant and *P. brassicae* DNA so the exact level of *P. brassicae* DNA extracted using each method is unknown.

Table 5.6. Comparisons of different DNA extraction methods at extracting good quality and high yielding DNA from *P. brassicae* infected roots of Chinese cabbage

DNA extraction method	Time taken for DNA extraction	Mean DNA yield dsDNA (ng/µl)	Quality of DNA
Boiling method (Tris-HCl pH 4.8)	30 minutes	906	Degraded DNA, poor quality
Boiling method (Tris-HCl pH 7.5)	30 minutes	53	Degraded DNA, poor quality
DNeasy	45 minutes	20	Single band of DNA, good quality
Fast Prep kit	40 minutes	18	Single band of DNA, good quality
Fast Prep Tris-HCl pH 7.5	40 minutes	638	Degraded DNA, poor quality
NucleoSpin	50 minutes	3	No band seen on gel
Puregene	2 hours 30 minutes	370	Single band of DNA, good quality
RED-Extract N-Amp Plant PCR kit	15 minutes	377	Degraded DNA, poor quality

***Plasmodiophora brassicae* DNA amplification using primers designed for the ITS region**

Each sample of DNA that had been extracted from *P. brassicae* infected Chinese cabbage roots using selected extraction methods were amplified by PCR using the nested primers designed by Faggian *et al.,* (1999) which had been previously shown to be effective at amplifying Australian and Swedish isolates of *P. brassicae* DNA from soil, water and dust (Faggian *et al.,* 1999; Wallenhammar and Ardwisson, 2001). This was to determine whether these primers would be effective at amplifying DNA from a British isolate of *P. brassicae* and to determine if all DNA extraction methods were as effective as each other.

In the first round of PCR using the outer primers PbITS 1 and PbITS 2, the DNA extracts from the NucleoSpin extraction kit, the DNeasy extraction kit and the boiling method using Tris-HCl at pH 7.5 had formed a band of approximately 1000 bp. The rest of the samples showed signs of primer dimer formation but no signs of *P. brassicae*

DNA amplification (Figure 5.1). Already after the first stage of PCR, *P. brassicae* DNA could be detected in some of the extraction samples. Nested PCR was then carried out to increase the sensitivity of the test using the PbITS 3 and PbITS 4 primers. After the nested step of the PCR reaction had been carried out on the samples, the expected DNA band length of 507 bp was seen in all samples, including the negative control samples which contained sterile de-ionised water in the place of DNA template (Figure 5.2). The results showed that the Faggian *et al.,* (1999) designed nested primers could successfully amplify British *P. brassicae* DNA extracted from Chinese cabbage roots using a wide range of extraction techniques but that DNA contamination could easily occur within the samples.

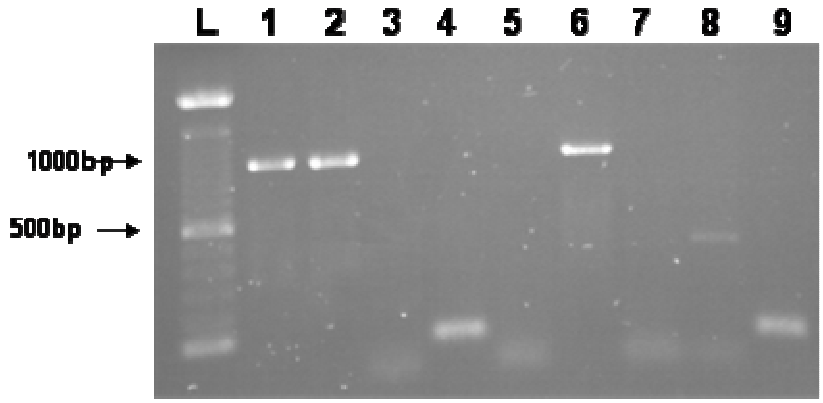

Figure 5.1. MegaFluor-stained gel of PCR amplified products from DNA extracted from *P. brassicae* infected Chinese cabbage roots with outer primers PbITS-1 and PbITS-2. L: 100 bp ladder; 1: Nucleospin; 2: DNeasy; 3: Tris-HCl pH 4.8; 4: Tris HCl pH 4.8; 5: Fast Prep kit; 6: Tris-HCl pH 7.5; 7: Puregene; 8: Puregene; 9: negative control.

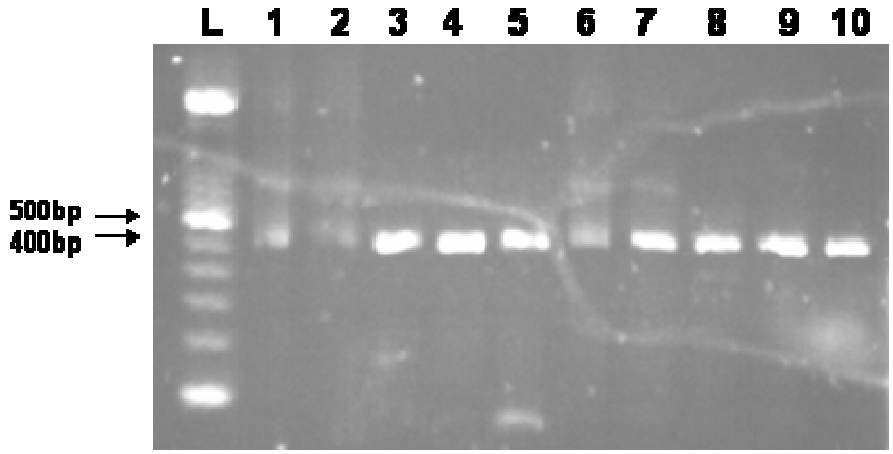

Figure 5.2. MegaFluor-stained gel of PCR amplified products from DNA extracted from *P. brassicae* infected Chinese cabbage roots with nested primers PbITS-3 and PbITS-4. L: 100 bp ladder; 1: Nucleospin; 2: DNeasy; 3: Tris-HCl pH 4.8; 4: Tris HCl pH 4.8; 5: Fast Prep kit; 6: Tris-HCl pH 7.5; 7: Puregene; 8: Puregene; 9: initial negative control; 10: nested step negative control.

Attempts to prevent contamination in the nested PCR reaction

To determine if the PCR components used in the reaction were causing bands in the negative controls due to being contaminated with *P. brassicae* DNA, the nested PCR assay was tested again using brand new PCR components that had never come into contact with *P. brassicae* DNA. Great care was also taken not to contaminate the negative control samples with *P. brassicae* DNA by keeping the negative control tubes far away from the positive control samples and by changing gloves during each stage of making the PCR master mix and when adding the samples to each tube. When these measures were carried out, there was no band seen in the initial negative control samples of the first round of PCR (Figure 5.3) but after the nested step of the PCR reaction had been carried out, the band was seen in the negative controls (Figure 5.4). This happened many times, even when all brand new PCR components were used and when only control samples containing just sterile de-ionised water were run in a reaction and no *P. brassicae* DNA had come into contact with any of the control samples.

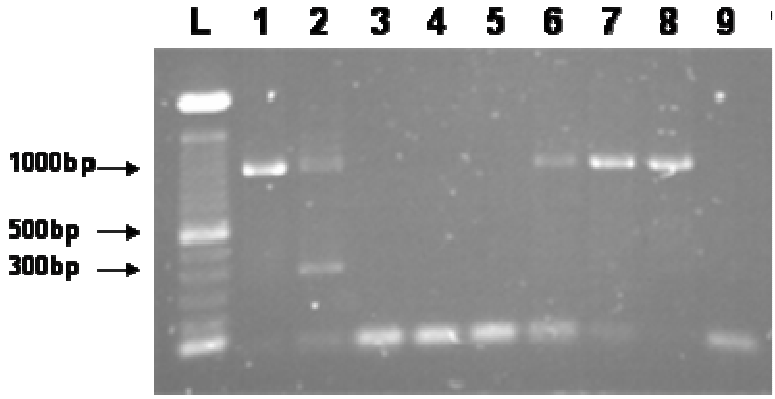

Figure 5.3. MegaFluor-stained gel of PCR amplified products from DNA extracted from *P. brassicae* infected Chinese cabbage roots with nested primers PbITS-1 and PbITS-2. L:100 bp molecular ladder; 1: Nucleospin; 2: DNeasy; 3: Tris-HCL pH 4.8; 4: Tris-HCL pH 4.8; 5: Tris-HCL pH 4.8; 6: Tris-HCL pH 7.5; 7: Puregene; 8: Puregene; 9: negative control.

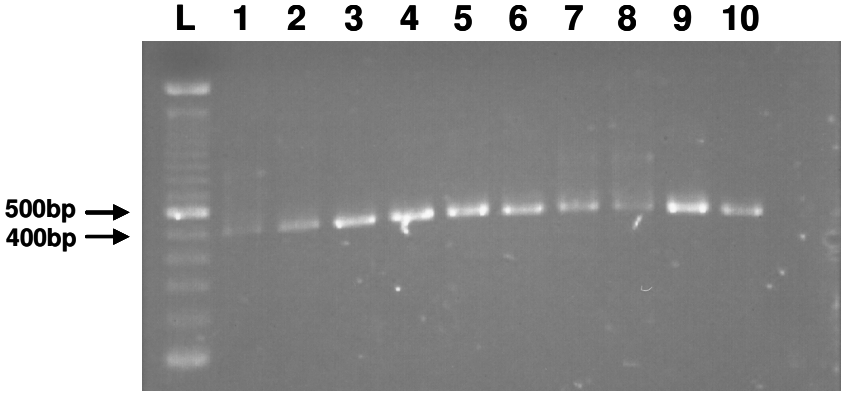

Figure 5.4. MegaFluor-stained gel of PCR amplified products from DNA extracted from *P. brassicae* infected Chinese cabbage roots with nested primers PbITS-3 and PbITS-4. L:100 bp molecular ladder; 1: Nucleospin; 2: DNeasy; 3: Tris-HCL pH 4.8; 4: Tris-HCL pH 4.8; 5: Tris-HCL pH 4.8; 6: Tris-HCL pH 7.5; 7: Puregene; 8: Puregene; 9: initial negative control; 10: nested stage negative control.

Altering PCR annealing temperature to avoid false positive results

The nested PCR step using the PbITS-3 and PbITS-4 primers was run using various annealing temperatures to determine whether optimising this step could eradicate the bands from the negative control samples. The results showed that if the annealing step temperature was raised to 68.4^{o}C or above, the bands seen in the positive samples and the negative samples would disappear. It would therefore not be achievable to keep the positive control bands while eradicating the negative control bands in this way.

Sequencing of the amplification product from the negative controls

To test if the band that was seen in the negative controls was *P. brassicae* DNA, the amplification product from one of the negative controls was cloned and the DNA was sequenced. By comparing the sequence with other sequences using the BLASTn program, it was seen that the DNA was indeed the expected 507 bp section of DNA that is amplified by the nested primers PbITS-3 and PbITS-4. This result showed that the band seen in the negative control samples had been caused by contamination of the samples with *P. brassicae* DNA.

Comparison of amplified P. brassicae DNA with other sequences of the same gene throughout the world

The 507 bp sequence of *P. brassicae* DNA was most closely matched with accession no: gi/30141009/djb/AB094980.1 in the BLASTn program. This was a *P. brassicae* gene for 18s rRNA, ITS1, 5.8s rRNA, ITS2, 28s rRNA. The isolate was from sak2, Japan.
There was 1 base pair difference between the sequence and the Japanese sequence. The sequence had a T instead of a C at base pair 294 compared to the Japanese isolate. A difference at this base pair section was also found in an English isolate. There were 7 base pair differences between the sequenced isolate and a Swedish isolate.

P. brassicae DNA amplification using primers designed for the isopentyltransferase gene

Due to the extreme sensitivity of the PbITS nested primers and the fact that altering PCR conditions could not eradicate the false positive results in the negative controls, these primers were considered not suitable for further quantitative work. Therefore, other sets

of nested primers that had been designed by Ito *et al.,* (1997) and Wallenhammer and Ardwisson (2001) were tested to see if they could also effectively amplify *P. brassicae* DNA from the root DNA extractions. Using the main primers PBAW 10 and PBAW 11 and the nested primers PBTZS 3 and PBTZS 4, the 398 bp band of *P. brassicae* DNA that would be expected using these primers was seen in all positive controls and there were no DNA bands in the negative controls (Figure 5.5). Using the outer primers PBAW 12 and PBAW 13, bands of the expected 1100 bp were seen in the positive control samples (Figure 5.6) and then when the nested primers were used in the next stage of the PCR reaction, only one band was seen in the positive control – the extraction technique that used the method of boiling the roots in Tris-HCl at pH 7.5 after crushing the roots in the FastPrep machine (Figure 5.7). These results would suggest that the PBAW 12 and PBAW 13 outer primers were not as effective at detecting *P. brassicae* DNA as the PBAW 10 and PBAW 11 primers when using different DNA extraction techniques.

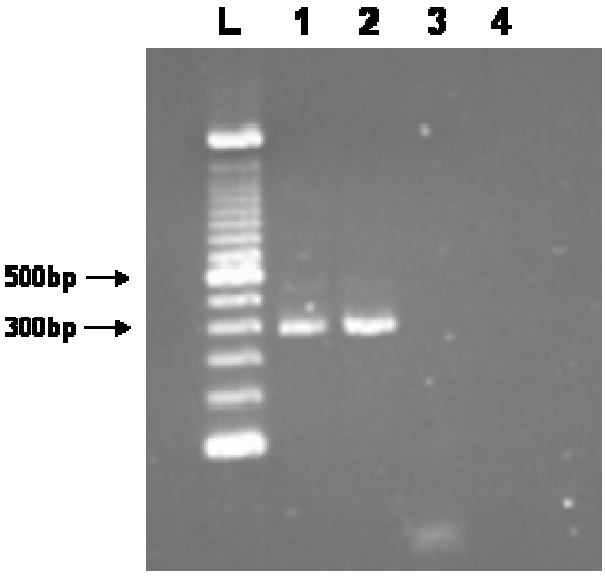

Figure 5.5. MegaFluor-stained gel of PCR amplified products from DNA extracted from *P. brassicae* infected Chinese cabbage roots having been amplified firstly using outer primers PBAW 10 and PBAW 11 and then nested primers PBTZS 3 and PBTZS 4. L: 100 bp ladder; 1: Fast Prep kit; 2: Fast Prep Tris-HCl pH 7.5; 3: initial negative control; 4: nested step negative control.

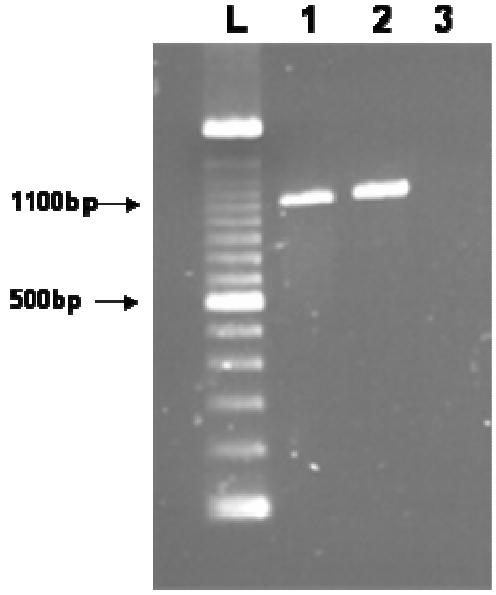

Figure 5.6. MegaFluor-stained gel of PCR amplified products from DNA extracted from *P. brassicae* infected Chinese cabbage roots with outer primers PBAW 12 and PBAW 13. L: 100 bp ladder; 1: Fast Prep kit; 2: Fast Prep Tris-HCl pH 7.5; 3: initial negative control.

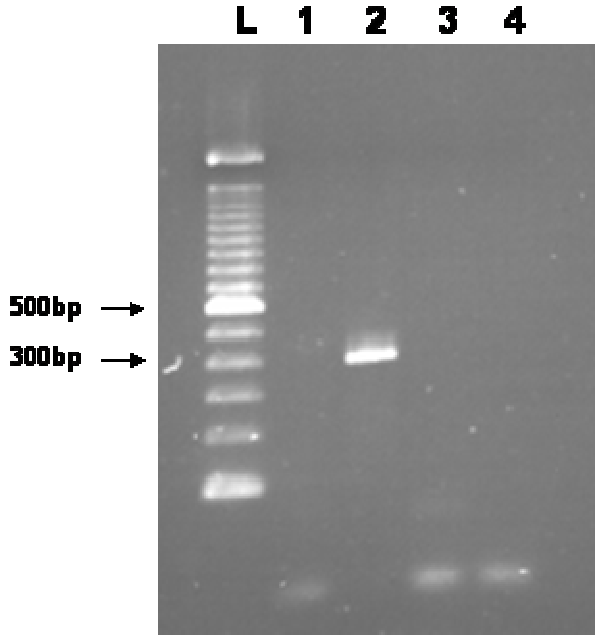

Figure 5.7. MegaFluor-stained gel of PCR amplified products from DNA extracted from *P. brassicae* infected Chinese cabbage roots with outer primers PBTZS 3 and PBTZS 4. L: 100 bp ladder; 1: Fast Prep kit; 2: Fast Prep Tris-HCl pH 7.5; 3: initial negative control; 4: nested step negative control.

DNA extraction and amplification from soil

John Innes No. 1 compost that had been artificially inoculated with 10^5 *P. brassicae* spores/g had its DNA extracted using the PowerSoil kit from MoBio. The kit was also used to extract DNA from a sample of naturally infested field soil from Lincolnshire. The results of using this extraction method to extract DNA from artificially inoculated compost and from naturally infested field soil can be seen in Table 5.7. The DNA was of high quality because a single bright band of DNA was visualized on an agarose gel. The DNA extracted from both soil samples was amplified using the PBAW 10 and PBAW 11 outer primers and PBTZS 3 and PBTZS 4 nested primers. A sample of DNA that had been extracted from *P. brassicae* infected Chinese cabbage roots was also amplified at the same time for comparison purposes. After the initial round of PCR, two bright bands of DNA could be seen; one corresponding to the root DNA extract and the other to the DNA that had been extracted from the artificially inoculated compost (Figure 5.8). The DNA amplified from the roots had a band length of the expected 774 bp. The DNA from the compost had a band of approximately 300 bp. When the nested stage of the PCR reaction was carried out, a bright band was seen in both of the positive control samples of the expected length of 398 bp (Figure 5.9). The DNA that was extracted from field soil using this kit also had its DNA successfully amplified using the same primers (gel not shown). These results demonstrated that *P. brassicae* DNA could be successfully extracted and amplified from artificially inoculated compost and naturally infested field soil using a commercial soil extraction kit and the nested primers designed for the isopentyltransferase gene.

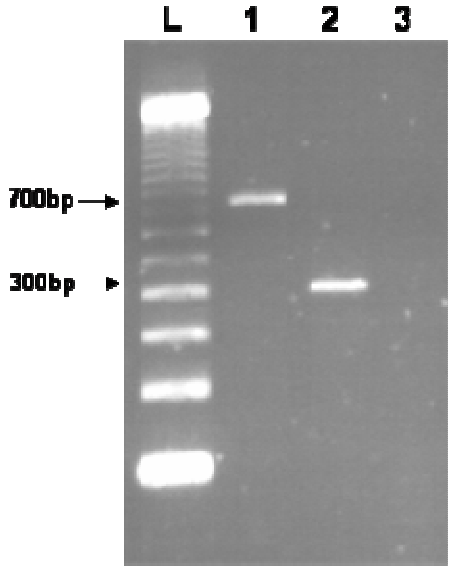

Figure 5.8. MegaFluor-stained gel of PCR amplified products from DNA extracted from *P. brassicae* infected Chinese cabbage roots and soil with outer primers PBAW 10 and PBAW 11. L: 100 bp ladder; 1: Fast Prep Tris-HCl pH 7.5; 2:: Powersoil (artificially inoculated compost; 3: negative control.

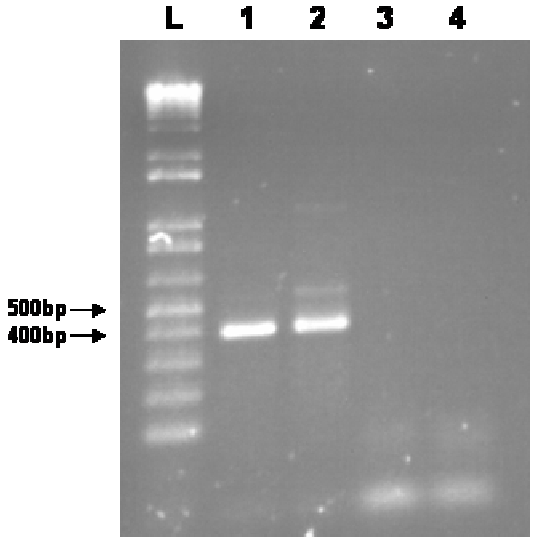

Figure 5.9. MegaFluor-stained gel of PCR amplified products from DNA extracted from *P. brassicae* infected Chinese cabbage roots and soil with nested primers PBTZS 3 and PBTZS 4. L: 100 bp ladder; 1: Fast Prep Tris-HCl pH 7.5; 2: Powersoil (artificially inoculated compost; 3: initial negative control; 4: nested stage negative control.

The powersoil kit extracted DNA from around 250 mg of soil. The PowerMax soil DNA extraction kit (Mo Bio) was used to extract DNA from 10 g of soil. Using this kit, DNA was extracted from three naturally infested field soils from Aberdeen, Fife and Lincolnshire. This kit was also used to extract DNA from John Innes No.1 compost that had been artificially inoculated with 10^5 spores/g soil. The amount of DNA extracted from each soil using the PowerMax kit can be seen in table 5.7. This DNA was seen to be of high quality when visualised on a gel. The Lincolnshire soil had the most DNA extracted from the soil using the PowerMax kit. The artificially inoculated compost had the least DNA extracted using this kit. The DNA extracted using this kit was amplified using the primers PBAW 10 and PBAW 11 and PBTZS 3 and PBTZS 4. *P. brassicae* DNA was successfully

Table 5.7. Comparisons of different DNA extraction methods at extracting high yielding DNA from *P. brassicae* infected soil.

	Extraction method and amount of DNA extracted (ng dsDNA/µl)		
Soil	PowerSoil	PowerMax	Retsch mill
Artificially inoculated John Innes No. 1 compost	6	2	74
Aberdeen	*	11	42
Fife	*	6	17
Lincolnshire	8	15	47

* These soils were not tested using the extraction methods due to unavailability of samples

Real-time PCR using the isopentyltransferase nested primers and SYBR green as a fluorescence detector

The PBTZS 3 and PBTZS 4 primers were tested in a real-time PCR assay to determine if it would be possible to use these primers in a quantitative real-time diagnostic test for *P. brassicae*. Samples of *P. brassicae* DNA that had been extracted from *P. brassicae* infected Chinese cabbage roots using 3 different DNA extraction methods were tested. All of the samples contained the same starting level of DNA (1 ng/µl). The results of the real-time PCR assay can be seen in table 5.8. The lowest C_t value was given by the Puregene DNA extract, the highest by the Tris-HCl pH 7.5 boiling method. The melt

curve also showed signs that primer dimer formation had taken place (melt curve not shown).

Table 5.8. C_t values of *P. brassicae* DNA extracted from infected Chinese cabbage roots and amplified with primers PBTZS 3 and PBTZS 4, detected using SYBR green.

DNA extraction method	Ct	Standard deviation
Negative control	N/A	N/A
Puregene	18.7	± 0.17
Tris-HCl pH 4.8	19.7	± 0.27
Tris-HCl pH 7.5	39.6	± 3.42

Real-time PCR using primers that had been specifically designed for use in a quantitative diagnostic test

Central Science Laboratory (CSL) York designed primers

DNA that had been extracted from both *P. brassicae* infected roots and soil were amplified in a real-time PCR assay using the primer pairs CSL1 and CSL2 and the specific probe CSL probe. All samples tested in the assay had a starting DNA level of 10 ng. The C_t values gained in this experiment can be seen in table 5.9. The results show that the primers and probe successfully amplified the DNA from all of the extraction samples. The C_t values for all of the samples were similar suggesting that the 10 ng of DNA added to the reaction all had a similar amount of *P. brassicae* DNA in them despite the DNA being a mixture of either spore and root DNA or spore and unknown soil microorganism DNA.

Table 5.9. C_t values of *P. brassicae* DNA extracted from infected Chinese cabbage roots and infested soil, amplified with primers designed by CSL, and detected with a specific probe.

DNA sample	Ct	Standard deviation
Negative control	N/A	N/A
Tris-HCl pH 7.5 (roots)	32	± 0.25
Fast Prep kit (roots)	28.9	± 0.62
John Innes positive control PowerMax	29.4	± 0.42
Aberdeen PowerMax	29.3	± 0.25
Fife PowerMax	29.6	± 0.35
Lincolnshire PowerMax	30	± 0.95

Real-time PCR on samples of DNA extracted from soil inoculated with spore-dilutions

DNA was extracted from John Innes No. 1 compost that had been artificially inoculated with a dilution series of *P. brassicae* spores. The extraction of DNA was done using the Restch mill method. This series of DNA extractions was amplified using real-time PCR by the CSL designed primers and probe. At the same time, a naturally infested field soil that was shown to cause a high level of disease in Chinese cabbage in a bait plant test and a field soil that had shown no signs of clubroot disease in a bait plant test had their DNA extracted using the Retsch mill method and these extractions were run alongside the extractions of the soils that had been artificially inoculated with known numbers of *P. brassicae* spores.

Four different sub-samples of each soil extraction were assayed and to evaluate the intra-assay variability, each sub-sample was assayed in triplicate. The mean C_t values given for each sub-sample can be seen in table 5.10. The results show that in the extracts with the lowest spore load, there were large differences between the C_t values of each sub-sample of soil the extraction. The C_t values of the spore dilutions did not show a strong pattern of lower C_t values as the spore load increased but the soil DNA extraction with the highest spore load did have the lowest C_t value. The field soil that had shown no sign of causing disease in brassicas had no amplifiable *P. brassicae* DNA in it. The field soil that had shown a high level of disease in Chinese cabbage was only shown to have amplifiable *P. brassicae* DNA in one sub-sample of the soil extract that was tested. The other sub-samples of this soil extraction gave no reading.

Table 5.10. Replicate C_t values of *P. brassicae* DNA extracted from soil, amplified with primers designed by CSL, and detected with a specific probe.

DNA extraction	Mean Ct				
	Sample 1	Sample 2	Sample 3	Sample 4	Total mean Ct; SD
10^3 spores/ g soil	34.9 ± 0.3	36.1 ± 0.1	24.0 ± 0.4	23.4 ± 0.8	29.6 ± 13.9
10^4 spores/ g soil	33.4 ± 0.7	33.2 ± 0.4	33.6 ± 0.6	33.4 ± 0.6	33.4 ± 0.5
10^5 spores/ g soil	33.4 ± 0.4	34.3 ± 0.6	33.1 ± 1.1	31.8 ± 0.1	33.2 ± 1.2
10^6 spores/ g soil	30.1 ± 0.3	29.7 ± 0.4	30.1 ± 5.2	29.0 ± 0.4	29.7 ± 2.8
10^7 spores/ g soil	26.6 ± 0.1	26.4 ± 0.2	27.4 ± 1.7	26.6 ± 0.7	26.7 ± 1.0
Positive field soil	*	30.8 ± 1.0	*	*	30.8 ± 1.0
Negative field soil	*	*	*	*	*

* No C_t readings were gained for these samples

CSL designed primers standard curve

To generate a standard curve for the CSL designed primers, the TaqMan C_t values were plotted relative to the corresponding serial dilutions of *P. brassicae* that had been added to the soil previous to it having its DNA extracted. The slope of the curve was -2.16 and the linear square regression coefficient was 0.873 (Figure 5.10). This was not statistically significant. A significant value would be greater than 0.950.

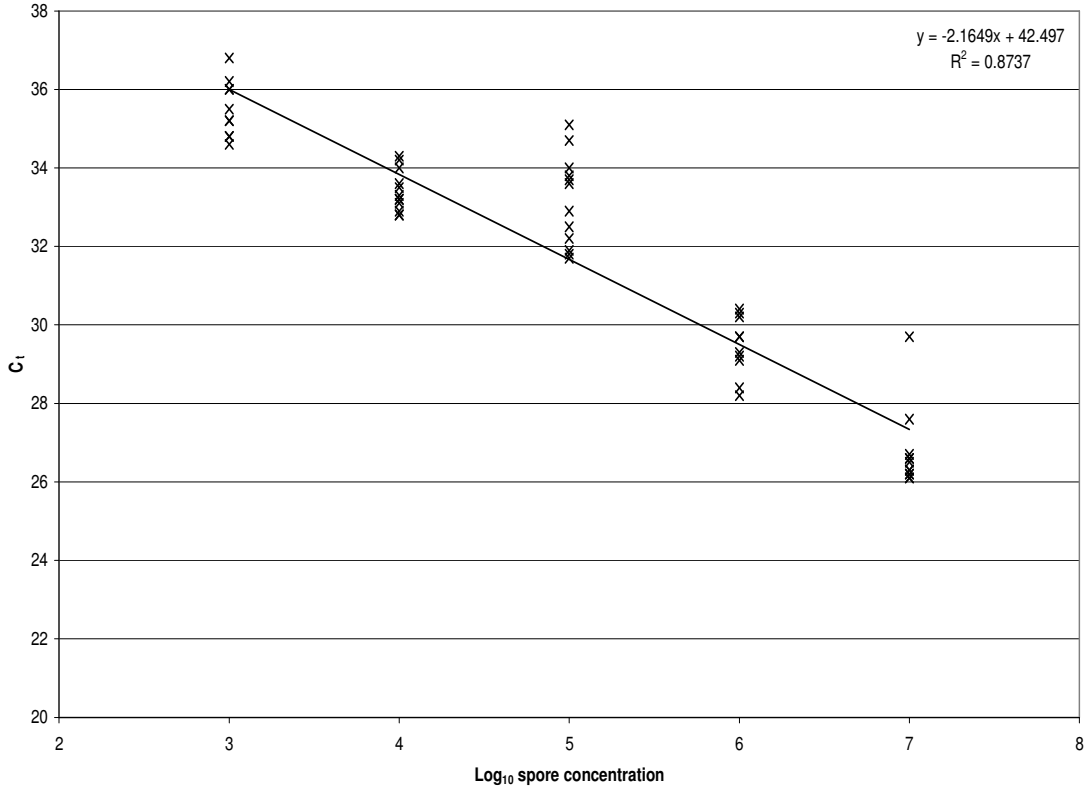

Figure 5.10. Standard curve showing the amount of spores present in the soil sample from which DNA was extracted versus the real-time PCR cycle threshold at which significant DNA amplification could be detected from that sample using the CSL designed primers and probe.

Faggian *et al.,* (2003) designed primers

Primers and a probe designed by Faggian *et al.,* (2003) were tested using the same soil samples used when generating a standard curve with the CSL designed primers (John Innes No. 1 compost with a serial dilution of *P. brassicae* spores added to them and a two field soil samples; one causing no clubroot disease in the field, the other causing a high level of disease). The C_t values for this assay can be seen in Table 5.11. The results show that there were large differences between the C_t values of each sub-sample of each soil extraction. The C_t values of the spore dilutions did not show a strong pattern of lower C_t values as the spore load increased but the soil with the highest spore load did have the lowest C_t value. The field soil that had shown no sign of disease in a bait plant test had no amplifiable *P. brassicae* DNA in it. The field soil that had showed

a high level of disease in a bait plant test was only shown to have amplifiable *P. brassicae* DNA in three sub-samples of the soil extract that was tested. The other sub-sample of this soil extraction gave no reading. A standard curve was also generated for these primers by plotting the C_t values relative to the corresponding serial dilutions of *P. brassicae* that had been added to the soil previous to it having its DNA extracted. This result can be seen in Figure 5.11. The slope of the curve was -1.16 and the linear square regression coefficient was 0.217 which was not significant.

Table 5.11. Replicate C_t values of *P. brassicae* DNA extracted from soil, amplified with primers designed by Faggian *et al.,* (2003), and detected with a specific probe.

DNA extraction	Mean Ct				
	Sample 1	Sample 2	Sample 3	Sample 4	Total mean Ct; SD
10^3 spores/ g soil	35.8 ± 0.8	18.5 ± 16.0	27.2 ± 1.2	27.5 ± 0.3	27.3 ± 9.4
10^4 spores/ g soil	34.1 ± 0.3	33.8 ± 0.5	30.2 ± 3.0	34.1 ± 0.2	33.1 ± 2.2
10^5 spores/ g soil	35.2 ± 0.2	35 ± 0.4	32.5 ± 0.2	33 ± 0.3	34.0 ± 1.3
10^6 spores/ g soil	30.6 ± 0.3	29.6 ± 0.3	28.7 ± 0.2	19.5 ± 16.9	27.1 ± 8.6
10^7 spores/ g soil	26.0 ± 0.1	25.9 ± 0.2	26.2 ± 0.3	17.4 ± 15.0	23.9 ± 7.5
Positive field soil	31.2 ± 1.3	27.1 ± 0.3	31.3 ± 0.6	*	29.9 ± 2.2
Negative field soil	*	*	*	*	*

* No C_t readings were gained for these samples

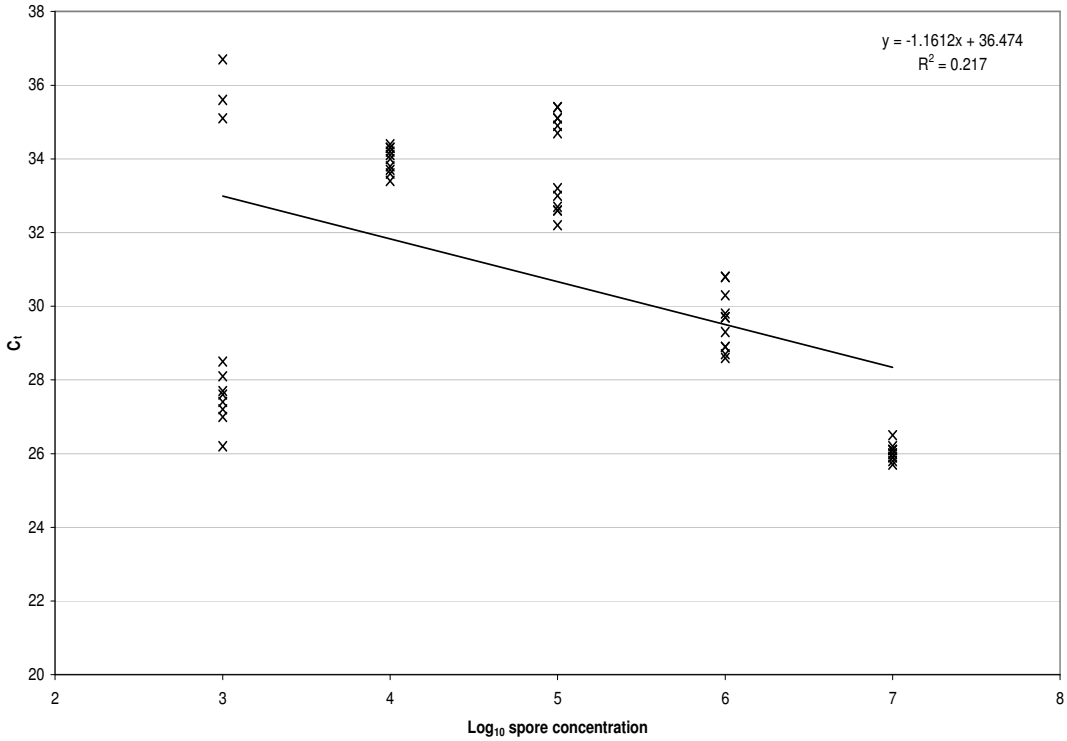

Figure 5.11. Standard curve showing the amount of spores present in the soil sample from which DNA was extracted versus the real-time PCR cycle threshold at which significant DNA amplification could be detected from that sample using the Faggian *et al.,* (2003) designed primers and probe.

Discussion

The development of a rapid, quantitative diagnostic test for the presence of *P. brassicae* DNA in soils would be of great use to brassica growers. While conventional nested PCR assays basically provide endpoint measurements suitable for qualitative yes/no results, the real-time TaqMan assay quantifies the starting concentration of spore DNA by monitoring the amount of reaction product during the exponential phase of the amplification cycles (Rondini *et al.,* 2003). The main advantage of real-time PCR over nested-PCR assays is that it is far less labour-intensive (only one PCR step, compared to at least two in nested-PCR). It is also performed in a closed system where post-PCR handling is not required so it reduces the risk of cross contamination because there is no transfer of amplified template from the primary to the secondary amplification reaction

and no transferring of the template onto agarose gel electrophoresis for the detection of PCR products. The results of the assay can also be obtained in only a few hours compared to a minimum of nine hours with nested PCR (Perandin *et al.,* 2004). The test can also be made quantitative with the use of standard curves containing known amounts of template DNA. Real-time PCR could be implemented in routine diagnostic tests and has the potential to provide growers with an idea as to the spore load within their soils, potentially within the space of one day. This would allow them to take specific clubroot control measures based on these results very quickly. Real-time PCR could also be used to develop a map of a field showing where 'hotspots' of *P. brassicae* spores were by carrying out quantitative real-time PCR on many soil samples taken from throughout the field. This would allow growers to apply treatments to specific parts of the field where they were required, hence reducing the costs of the addition of the treatments. Real-time PCR is the most precise method available for gene quantification (Cankar *et al.,* 2006). Its performance however is substantially influenced by the sample properties.

When developing a quantitative PCR test for an obligate soil-borne pathogen like *P. brassicae*, many different factors have to be taken into account. Successful quantification depends crucially on the quality of the sample DNA analysed (Cankar *et al.,* 2006). The quality of DNA concerns not only the purity but also the structural integrity of DNA obtained and the term PCR forming units (PFU) was introduced to distinguish between structurally intact and damaged (non-amplifiable) target DNA copies (Cankar *et al.,* 2006).

Plasmodiophora brassicae DNA in soil can be present in low amounts due to the patchy nature of the distribution of the spores in the soil. Many substances in soil such as plant secondary metabolites can also affect PCR amplification of samples which could also influence the reliability of the quantification. The extraction method used in the test must therefore ensure a high yield and quality of the DNA obtained, must remove PCR inhibiting substances, and must also be carefully selected, since even components of DNA extraction solutions can influence PCR reactions (Cankar *et al.,* 2006). The

primers used in the test must also be highly specific to *P. brassicae*, as soil DNA extractions will contain the DNA of many organisms including closely related organisms to *P. brassicae* such as *Spongospora subterranea* (Faggian *et al.,* 1999). If the primers are not specific, an over-estimation of the numbers of spores present in the soil would be made. They also must not be over-specific otherwise only specific pathotypes of the organism could be amplified, hence giving an under-estimation of spore loads in the soil.

Effectiveness of nested primers at amplifying *P. brassicae* DNA from roots

DNA that is extracted from plant tissue may contain many PCR-inhibiting substances such as polysaccharides and pigments that can lead to poor PCR amplification and can make samples unsuitable for both conventional PCR and quantitative PCR analysis (Cankar *et al.,* 2006). Therefore, to test the ability of primers at amplifying *P. brassicae* DNA from plant tissue in these experiments, many different methods of DNA extraction were used. The DNA extracted from the roots would have contained a high level of plant DNA so the exact levels of *P. brassicae* DNA extracted using each method was unknown, but it was seen that the commercial DNA extraction kits were good for extracting high quality DNA but not all gave high yields. A cheaper and faster method of DNA extraction such as boiling the roots in Tris-HCl buffer at certain pH levels yielded high levels of DNA but the DNA was degraded and not of as high quality as the DNA extracted using kits. Obtaining high quality DNA is especially necessary when using quantitative PCR because a degraded piece of DNA would represent the presence of *P. brassicae* but it may not be amplified. This would lead to an under-estimation of the actual spore level present. DNA extracted from the infected roots using every method used was capable of being amplified by the primers tested. These experimental results have therefore shown that an inexpensive and fast method of DNA extraction was just as effective at providing DNA with good enough quality for use in PCR as more expensive or more time-consuming commercial kits. If the test was being developed to give just a yes/no answer as to the presence of *P. brassicae* DNA in roots, a cheap and fast method of DNA extraction such as boiling the roots in Tris-HCl buffer would be

sufficient to provide this answer, but it would not be effective for quantifying DNA levels.

Amplification of *P. brassicae* DNA using nested primers designed by Faggian *et al.*, (1999)

The set of nested primers designed by Faggian *et al.,* (1999) were designed to detect the ITS1 (internal transcribed spacer), 5.8S and part of the 18S and ITS2 regions of *P. brassicae*. This region has been useful for detecting other microorganisms such as *Rhizoctonia solani* (Lees *et al.,* 2002) because the rDNA region can be present in up to 200 copies per genome (Faggian *et al.,* 1999) making the primers extremely sensitive at detecting the DNA. Using the outer primers PbITS1 and PbITS2, Faggian *et al.,* (1999) showed that an amplification product was detectable using only 1 pg of purified DNA template. When the corresponding nested primers were used, this detection limit was reduced to 0.1 pg of DNA template. These primers had been rigorously tested against other microorganisms and against plant DNA and it was shown that they did not amplify plant DNA or other closely related organisms such as *S. subterannea,* or unidentified bacterial species that were present in *P. brassicae* resting spore suspensions Faggian *et al.,* (1999). The primers were also tested by Faggian *et al.,* (1999) on DNA extracted from soil, water and plant material and against many races of *P. brassicae* and were shown to be specific and highly sensitive. These primers would therefore be thought of as being useful in the development of a diagnostic test for the detection of *P. brassicae* in soils. The experimental results seen in the experiments carried out in this study showed that bands of DNA were present in the nested step of the PCR but not always in the initial step when these nested primers were tested with different root DNA extracts. This demonstrated that the nested PCR step in the reaction was essential when trying to detect low levels of DNA in samples. There was a problem with using these nested primer pairs because bands of DNA could be seen in negative control samples where no *P. brassicae* DNA had been added. Despite trying to minimise any possibilities of contamination in the samples and by altering PCR cycling conditions to try and minimise any false-positive results, the extreme sensitivity of these primers was determined to be a problem because contamination was nearly always present in the

negative controls after the nested PCR step. If these primers were used in a diagnostic test, there could be a high possibility of a very small amount of contamination in the PCR tubes leading to a false-positive result which would suggest that a sample contained *P. brassicae* when it did not.

***P. brassicae* ITS sequence in negative control samples**

The DNA band seen in the negative controls when using the Faggian *et al.,* (1999) designed ITS primers was sequenced and was found to be *P. brassicae* DNA confirming the fact that contamination had taken place. A laminar flow cabinet had been used to minimise the risk of contamination so *P. brassicae* spores in the air would have been an unlikely source of contamination. The contamination could have come from carryover from other samples. The level of contamination would only need to be minimal for a band to have been detected in the negative controls due to the sensitivity of the nested primers. The fact that many components were added to a master mix in the PCR reaction could also have added to the amount of times contamination could have taken place. Putting the amplified DNA samples onto a gel to visualise the bands could also have been a source of contamination. Again, this shows that real-time PCR has advantages in reducing the possibilities of contamination compared to nested PCR as the amount of handling of the samples is reduced.

The sequence of the amplified section of *P. brassicae* DNA was 1 base pair different to a Japanese isolate and 7 base pairs different to a Swedish isolate described in the BLASTn database. This suggests that the ITS region is generally well conserved among isolates throughout the world but that British and Japanese isolates are more similar to each other than to the Swedish isolate. Sequence differences indicate that there is variation in *P. brassicae* populations, and the knowledge of these different sequences could be useful when studying the diversity of *P. brassicae* throughout the world. PCR using these primers could therefore help with pathogen population studies and could also help with resistance breeding programs. Using a molecular method to detect different *P. brassicae* pathotypes may be more beneficial than using the ECD method of using

differential plant hosts (described in Chapter 2) because it would be more likely to discover a wider range of pathotypes present within a soil sample compared with plant hosts. A molecular test would also be more rapid and would require fewer resources compared with growing plants in a glasshouse.

Amplification of *P. brassicae* DNA using pimers designed for a suspected isopentyltransferase gene

Ito *et al.,* (1997) designed a set of primers that amplified a 398 bp fragment of a suspected isopentyltransferase gene of *P. brassicae*. This gene is a single copy gene which means that these primers are not as sensitive at detecting DNA as ITS primers which are present in multiple copies within a genome. This was shown by the fact that Wallenhammer and Ardwisson (2001) used these primers and could amplify *P. brassicae* DNA when the template was diluted to 260 ng. This is not as sensitive as the first round of PCR using the Faggian *et al.,* (1999) designed primers. Wallenhammer and Ardwisson (2001) designed outer primers for the Ito *et al.,* (1997) designed primers to increase the sensitivity of the test, and by carrying out a nested PCR using both sets of these primers, the sensitivity of the detection increased to 260 pg. Wallenhammer and Ardwisson (2001) demonstrated that these primers could detect *P. brassicae* in different soil types but could not reliably detect *P. brassicae* corresponding to a soil sample with a level of infection corresponding to a disease index of 8 in a bioassay. When these primers were used in this study to examine their effectiveness at amplifying *P. brassicae* DNA from infected Chinese cabbage roots, the combination of the outer primers PBAW 10 and PBAW 11 with the nested primers amplified *P. brassicae* DNA from all DNA samples tested. The combination of the outer primers PBAW 12 and PBAW 13 with the nested primers only amplified some of the DNA samples. This would suggest that the PBAW 10 and PBAW 11 primers were more compatible with the nested primers than PBAW 12 and PBAW 13 and were therefore more sensitive at detecting low amounts of *P. brassicae* DNA. There was never any contamination seen in the negative controls when using these primers. This could be because the primers were not so sensitive at detecting low amounts of DNA and so if there was only a small amount of

contamination in the negative controls, it would not be amplified by these primers. These primers may not be able to detect DNA at as low a level as the Faggian *et al.,* (1997) designed primers, but disease generally only occurs if the spore level is above 1000 spores/g soil (Wallenhammer, 1996). The isopentyltransferase gene primers may therefore be able to detect *P.* brassicae DNA corresponding to this level and would give an indication that a possibility of disease would occur.

Development of a diagnostic test by extracting *P. brassicae* DNA directly from soil

For an effective quantitative diagnostic test to be developed for *P. brassicae*, it would be preferable if the *P. brassicae* DNA was extracted directly from soil. This is because if the DNA is detected directly from soil, *P. brassicae* would be able to be detected in potentially one day and the initial level of spores present within the soil could be determined without them being amplified within a plant. This would give a better estimation of the initial spore level in the soil because the amount of spores that enter a plant do not always correspond to the level of spores that are in the soil. Some spores may be more infective than others, and then one spore can lead to the formation of differing amounts of zoosporangia, each containing a different amount of zoospores within the root (Asano *et al.,* 2000). This would mean that it might be difficult to compare the level of *P. brassicae* DNA in the roots to the initial soil spore load. A small number of spores can also give rise to the same level of disease in a host as a large number of spores if the conditions for infection are optimal in a bait plant test (Colhoun, 1953). The detection of *P. brassicae* DNA directly from soil would also not rely on glasshouse space or environmental conditions, and the cost of carrying out the test would be lower because it would avoid the need for growing plants and would only involve the DNA extraction and PCR amplification stages.

The collection and extraction of a representative soil sample is of primary importance when developing a diagnostic test that would determine the amount of a particular organism in the soil. Clubroot spores are known to have a patchy distribution throughout field soil and spores are generally found in waterlogged ditches (Monteith,

1924). Therefore, the sampling method is important because if only some areas in a field contain clubroot spores, sampling of the soil could easily miss these patches. In the bait plant test of the Scottish Agricultural College, a soil sample of 2 kg is recommended. This is sampled by a consultant using a statistically devised method (SAC, SOP: OSR 007), and this type of soil sampling may provide a reasonable sample of soil for use in the real-time PCR test. Increasing the size of the soil sample that the DNA is extracted from should improve the yield of DNA from field soils and could help improve the detection in fields with low levels of spore inoculum, however, DNA is generally only extracted from a small sub-sample of soil because of the limited techniques of extracting DNA from soil that are available.

Different methods of soil DNA extraction were tested in these experiments because the DNA extraction method can substantially influence the quantification of soil DNA levels. This is because different methods differ in their effectiveness in removing substances that interfere with the PCR reaction such as polysaccharides, proteins, phenolic compounds and other secondary metabolites. In addition, components of the DNA isolation solutions can themselves influence PCR reactions. Therefore, the DNA extracted from different soil types using different methods must be evaluated for suitability in quantitative analysis and the appropriate choice of an extraction method suitable for a particular soil type must be made for successful downstream analysis. The extraction method must provide a sufficient level of DNA to guarantee reliable detection of the minimum level required for disease and the DNA must also be of high quality because as mentioned, amplifiable DNA is needed for accurate quantification to occur. The choice of extraction method is often a trade off between costs, optimal yield of DNA and removal of substances that could influence the PCR reaction.

Extraction of *P. brassicae* DNA from different volumes of soil

The commercial kit (PowerSoil, Mo Bio) that extracted DNA from 0.250 g of artificially inoculated compost and naturally infested field soil successfully yielded good quality DNA and the extracted DNA was also amplified using primers specific for *P. brassicae*

showing that *P. brassicae* could be detected in an extremely small quantity of a sub-sample of soil. Despite this fact, using this kit for a diagnostic test would not be desirable because of such a small sample of soil used as clubroot spores are very patchily distributed within a field environment and extracting DNA from 0.250 g of soil is not likely to be representative of a field environment. Spores could easily be missed during the sub-sampling process for this kit and the risk of saying that a field did not contain *P. brassicae* spores when it did is likely to be high if such a small soil sample was used.

Another commercial kit for soil DNA extraction was also tested (PowerMax soil, Mo Bio) and this kit extracted DNA from 10 g of soil. This would increase the chances of any spores in the soil being detected compared to the kit that extracted DNA from only 0.250 g of soil. This kit also yielded quality DNA from different types of soil and *P. brassicae* DNA was detected from all soils using this kit. This kit would be preferable to the other kit because of the amount of soil used which may increase the chance of *P. brassicae* DNA being detected, but it is more expensive.

Commercial kits are expensive and have limits to the amount of soil from which they can extract DNA, but a DNA extraction method like the one that is used in conjunction with the Retsch mill can be applied to large volumes of soil and is cheaper than the kits. DNA extracted from as large a volume of soil as is possible would be necessary in the development of a diagnostic test because a larger soil sample would be more likely to be representative of field soil than a small soil sample. The Retsch mill that was used in these experiments had the capability of extracting DNA from 60 g of soil. DNA was extracted from different field soils using this method and also from John Innes No.1 compost that had been inoculated with different levels of *P. brassicae* spore inoculum. The DNA was not of as high quality as the commercial kits, but as was seen with the Tris-HCl method of extracting DNA from roots, DNA extracted using this method was still capable of being amplified using selected primers. The use of PVPP columns to reduce the level of PCR inhibitors from the soil appeared to be effective and this method

may be a good way of extracting DNA from soil to be used in downstream PCR applications.

Real-time PCR using Ito *et al.*, (2001) designed primers and SYBR Green

Plasmodiophora brassicae DNA extracted from plant roots was amplified using the isopentyltransferase gene nested primers with SYBR green fluorescent dye in a real-time PCR reaction. The DNA was amplified using these primers but the formation of primer-dimers was also suspected. This would suggest that these primers were not optimal for the detection of *P. brassicae* under quantitative real-time conditions because the formation of primer-dimers would reduce the efficiency of the reaction. SYBR green is not a specific probe and detects the amplification of any source of DNA. With primer dimers being formed, this could give an inaccurate reading of the DNA levels present.

The Tris-HCl extraction method had a higher C_t than the other extraction methods tested in this real-time PCR assay despite the fact that the same amount of DNA had been added in each reaction. This could suggest that the Tris-HCl method had a high ratio of plant to *P. brassicae* DNA compared to the other extraction methods and so there was less *P. brassicae* DNA in the initial sample. It could also suggest that because the Tris-HCl boiling method does not use lots of DNA purification steps, there could have been other substances that were co-extracted with the DNA which inhibited the PCR reaction. The effect of co-extracted substances can result in the blocking of amplification completely, or the C_t values obtained in the PCR reaction could be higher than expected from the number of target molecules added to the reaction, or a decrease of overall efficiency of PCR reaction could be observed. The DNA extracted using the Tris-HCl method was also degraded and as suggested, in a test that involves quantification of DNA, degraded DNA may not be amplified as efficiently as high quality DNA. All of these factors would cause under-estimation in the level of spores present in the soil. The results of this experiment demonstrated that choosing the optimum method of DNA extraction when carrying out a quantitative real-time PCR test is essential if it is necessary to accurately estimate the number of spores present in a sample.

Real-time PCR using CSL and Faggian *et al.*, (2003) designed primers and probes

Probes that are specifically designed to detect an organism are of more use in a quantitative test compared to the non-specific SYBR green probe. They are less likely to be affected by co-extracted DNA of other microorganisms. CSL had developed primers and a probe specific for *P. brassicae* DNA and kindly allowed them to be used in these experiments. CSL have used their primers for detection of clubroot from plant tissue. Their method involves growing Chinese cabbage in the soil that is to be tested, and allowing root hair infection to take place which takes one to two weeks. They extract DNA from roots and use the primers to detect and quantify the presence of *P. brassicae* DNA within the roots. This test therefore still takes some time and does not really give a true indication of the level of spores in the soil because as mentioned, the number of spores in a plant does not always correspond to the number of spores in the soil (Asano *et al.,* 2000). Their method only detects viable spores however, and this may be an advantage of this method of detecting spores using real-time PCR than extracting DNA directly from soil.

In the experiments in this study, the CSL designed primers successfully amplified *P. brassicae* DNA that had been directly extracted from John Innes No. 1 compost that had been inoculated with different levels of *P. brassicae* resting spores using the Retsch mill method. The primers also amplified DNA that had been extracted from a naturally infested field soil which gave a high level of disease incidence in the field, and they did not detect DNA in a field soil that had been deemed to be clubroot free after a bait plant test. This suggests that it would be possible to extract *P. brassicae* DNA directly from soil and amplify it without having to use a bait plant step when using these primers. It also demonstrated that the primers and probe can detect *P. brassicae* DNA in fields that have a high level of disease incidence. The same results were seen when using the Australian primers and probe that had been specifically developed for *P. brassicae* with the same soil samples, also suggesting that these primers and probe could be used to detect and quantify *P. brassicae* DNA directly from soil.

Standard curves

Spores can potentially be quantified using a standard curve produced by dilutions of standard reference materials. A standard curve was created for each primer pair used in the real-time PCR assays based on the DNA extractions from compost that had been artificially inoculated with a serial dilution of spores. Both sets of primers did not give a statistically significant standard curve although the CSL curve was more significant than the Australian curve because it had a higher R^2 value. There was a greater difference in C_t values in soil sub-samples for the lower levels of spores in the soil than the higher levels of spores in both of the standard curves. This would suggest that the quantification of spores in soils with high levels of inoculum would be more accurate than the quantification of low spore loads. A lot of variation is present within individual soil types and the sub-sampling of the soil could cause initial differences in the starting level of DNA to be amplified. This would be especially true in soils with low levels of spores as spores may not be present in a small sub-sample of soil. This could also be a reason for the closeness in C_t values of the soils with lower spore loads added. The efficiency of amplification is also not necessarily influenced in the same way for all DNA samples. This fact could lead to even larger errors in quantification.

The *P. brassicae* DNA present in natural field soil was not as effectively detected using the CSL designed primers compared with the Australian primers. This may have been because of the soil samples used in the tests. *P. brassicae* DNA may only have been present in one of the soil sub-samples tested with the CSL primers and three of the sub-samples tested with the Faggian *et al.,* (2003) designed primers. These results demonstrated that there is a need for lots of replication within the test to ensure that low levels of *P. brassicae* DNA are successfully detected.

When comparing the C_t values for the naturally infested field soil, both sets of primers gave a reading of around 30. This would suggest that both primer sets were acting similarly in their ability to amplify *P. brassicae* DNA. The standard curves did not give

a significant regression value and so comparing the naturally infested field soil value with the standard curve was not justified.

The similarity of PCR efficiency for the unknown sample and the standard reference material is a perquisite for exact quantification of the sample (Cankar 2006). Differences in the PCR efficiencies between the reference material and the sample can lead to under- or over-estimation of spore content. Therefore, the main problem with the real-time PCR assays that were carried out in this study was that the DNA extracted from the field soil containing an unknown level of spores was being compared with artificially inoculated John Innes compost. Different soil types can contain different soil inhibitors and so comparing a field soil with a compost may not give an accurate quantification of spores in the soil.

Also in this experiment, the level of spores in the naturally infested field would be calculated from a regression line of C_t values obtained from dilutions of DNA extracted by the Retsch mill method. The Retsch mill method was shown to provide degraded soil DNA. If high DNA quality is used in some reactions and low quality DNA used in others, high quality DNA samples would be biased and would lead to the overestimation of spore content in the soil. Therefore, the estimation of spores in the soil is highly influenced by the DNA extraction method used. This would mean that in the diagnostic test, all samples would have to have their DNA extracted using the same method as was used for creating the standard curve.

DNA extraction from different soil types to create standard curves for each soil type

Some soil types have high humic content which could hinder PCR amplification of DNA. Spores may adhere to the soil particles of different soil types and not be properly extracted using various different soil DNA extraction methods. Therefore, different extraction methods may be necessary for extracting DNA from different types of field soils to give optimal DNA extraction. DNA extraction methods could therefore be defined that are most suitable for removal of potential substances that could affect PCR

amplification and are suitable for those soils that contain them. Different soil types could also have spore dilutions added to them and have their DNA extracted to create standard curves for each soil type. Soils of a similar type could then be compared with each other to give a more accurate estimation of resting spores in the soil.

Bait plant tests to compare field disease level to DNA level seen in real-time PCR

Different soil types can facilitate or hinder clubroot disease (sandy soils are less conducive to disease than soils with high humic content (Murakami *et al.,* 2000)) so the amount of spores present in the sample may not always correspond to disease level seen in the field environment. Therefore, standard bait tests on different types of soil could also be useful so that actual disease levels seen on plants when different levels of spores are added to the soil could be compared to DNA levels seen in real-time PCR to give a clearer idea of possible disease incidence that will be seen in the field. A problem that may be encountered with this method is that autoclaved soil with different amounts of spores added usually gives less disease incidence than non-autoclaved soils (shown in an experiment in Chapter 3 and by Murakami *et al.,* (2002) and Colhoun (1953). Different environmental conditions in the field also cause a different level of disease in the plants compared to what is seen under glasshouse conditions (Colhoun, 1953). These are all factors which would make it difficult to accurately determine the level of disease that would be present in a field soil when measuring the initial level of spores present in a soil sample.

Currently, little information on the performance of real-time PCR on different soil types is available (Cankar 2006) and this may be a good area for future research in the development of a *P. brassicae* quantitative test.

Presence of viable/non-viable spores in soil

In the case of *P. brassicae* resting spores, DNA could be detected in a sample using real-time PCR but the DNA may not be from viable resting spores. This would mean that a positive result in a sample would not necessarily correspond to a certain disease level in

the field. Faggian *et al.,* (2001) carried out experiments where non-sterile soil was spiked with non-viable resting spores and then sampled the soil at weekly intervals for a month to determine *P. brassicae* DNA levels. PCR positive results for *P. brassicae* DNA were obtained for the entire sampling period, but a trend of diminishing product with time was seen, suggesting that biological processes were eliminating the non-viable spores. This again demonstrates that the development of a quantitative real-time PCR test is difficult because the level of DNA detected may not relate to viable spores and the level of viable spores able to cause disease would be difficult to determine. If a way of estimating the average number of non-viable spores in a sample could be developed, this would help in the development of the quantitative test.

Other uses of real-time PCR in clubroot research

The use of real-time PCR would be useful to not only examine the spore load of *P. brassicae* in the soil, it would also be useful to look at pathogen populations due to the differences in the ITS region of different races of the spores. Real-time PCR could also be used to look at the level and types of other microorganisms present in the soil along with *P. brassicae*. Soil microflora is observed to have a prominent effect on the ability of *P. brassicae* to cause disease, so it would be interesting to follow this line of research in the future to give a better understanding about the effect of different microorganisms at promoting or preventing disease which at the moment is currently unavailable.

Conclusion

The development of a rapid, accurately quantitative real-time PCR test for the presence of *P. brassicae* directly from soil would of great value to brassica growers. It would enable brassica growers to discover much more quickly if their fields are infected with clubroot and would allow them to apply appropriate control treatments for the spore loads present in their soil or allow them to avoid fields with high inoculum levels. This would help brassica growers increase their yields and therefore maximise their profits.

These experiments have demonstrated that *P. brassicae* DNA can be successfully extracted and amplified from 0.25 g of soil to 60 g of soil using different DNA

extraction methods and that *P. brassicae* DNA can be extracted and amplified from both artificially inoculated compost and naturally infested field soil using sets of specific primers and probes designed specifically for the ITS region of the genome.

The experimental results have also shown that there are many problems when developing a quantitative test based on extracting DNA directly from soil. Firstly, a soil sampling method that has been statistically developed to take into account the patchy nature of clubroot spores within a field would be needed to provide a sample that would accurately reflect the spore loading in the test field. DNA may have to be extracted from a large volume of soil so that the possibility of detecting the presence of spores within a soil sub-sample would be increased. There would also need to be replication within the tests due to the differing results in DNA quantification between soil sub-samples.

The choice of DNA extraction procedure was shown to influence the ability of the test to quantify spores. The extraction method selected would have to provide a high yield and good quality DNA for the spore level in the soil to be quantified accurately. The choice of extraction method would have to involve a trade off between costs, optimal yield of DNA and removal of substances that could influence the PCR reaction. Different DNA extraction methods for different soil types may need to be developed for this reason.

The primers and probes used in the test would have to be specific and sensitive to *P. brassicae* so that interference from other DNA would not affect the quantification of spores. Of the primers tested, the primers designed by CSL were shown to provide the most significant standard curve and may be used in the future development of the test.

To quantify the levels of spores and make the results relative to the level of disease that would occur in a field, the development of standard curves relating spore load to different soil types could be developed. These standard DNA curves could be compared to disease level seen in the field to make the tests more relevant to the field environment. There may be too many variables for this to be carried out accurately so the use of

known bioassay methods may provide an answer to this problem. This would have to be a major area of research in the development of a quantitative diagnostic test. The knowledge that non-viable spores may also be present in the soil and could be amplified using the primers also has to be taken into account in relating spore loads measured to disease incidence seen and this could be an area of future research. The results achieved in these experiments are promising for the development of a quantitative test for clubroot spores directly from soil and the factors that make the development of the test more difficult could be researched further to help develop a test that would be relevant to a field environment.

CHAPTER 6 - GENERAL DISCUSSION

Current control measures for clubroot such as crop rotation and liming to raise soil pH are not adequate because they are inconsistent in their ability to reduce disease. Vegetable brassica growers require a range of sustainable and effective methods to allow them to manage clubroot successfully. The aim of this study was therefore to examine and refine the current methodologies for clubroot control and exploit new approaches for use in the field to reduce spore loads or protect young transplants against disease.

One of the key features for developing an effective control approach would be to provide an accurate estimation of the problem in the soil. Current methods for detecting the presence of *P. brassicae* spores in soil include a bait plant test which allows the detection of high spore loads in the soil but which is slow (it takes six weeks to complete) and uses lots of glasshouse space. An ELISA test may be able to detect low spore loads in the soil but this type of test suffers from cross-sensitivity with closely related soil microorganisms (Donald *et al.,* 2006). Therefore, there was a need for a new specific, rapid and accurate test for detecting and quantifying the spore load in the soil to be developed. The knowledge of the spore load in the soil would help brassica growers to select cost-effective treatments for controlling the specific level of disease in the soil. For example, low spore loads may only require a lime treatment to provide control whereas a high spore load may require a fungicide.

Within this study, an attempt was made to develop a real-time PCR diagnostic tool that would quantify the number of *P. brassicae* spores directly from soil. *P. brassicae* DNA was successfully extracted and amplified from naturally infested soil and from artificially inoculated compost. However, this work only scratched the surface of what would be required to create an accurate diagnostic test that would quantify spores from field soil. The creation of standard curves to compare known spore loads in the soil with the amount of DNA extracted was observed to be difficult to develop in this study. Low spore loads in artificially inoculated compost were unable to be quantified accurately. However, a set of primers and probe designed by CSL York were determined to be the most suitable for future diagnostic test development. This view is based on the standard

curve determined when using these primers to quantify known spore loads extracted from soil. This is because this standard curve was more significant than another standard curve determined using a different set of primers and therefore the CSL primers would give a more accurate spore level estimation than for the other sets of primers that were tested. However, the use of these primers for detecting low spore loads in many different natural soil types may be limited as they did not detect spores in every sub-sample of naturally infested field soil tested.

Problems in developing a quantitative PCR test using real-time PCR were highlighted in this study. PCR would be able to detect non-viable spores and therefore the test would provide an over-estimation of the spore load in the soil. However, low quality DNA would provide an underestimation of the spore load. The development of this PCR-based test for diagnostic purposes would therefore require further research. Different methods of soil DNA extraction may be needed for specific soil types to yield maximum levels of high quality DNA for use in the PCR reaction, and standard curves may have to be created for each soil type for appropriate comparisons between DNA extracted from each soil type and the amount of spores present within the sample. The development of this test would have to focus on relevance in the field. This would have to include the development of appropriate soil sampling methods to evaluate the level of *P. brassicae* spores found in the sample. The level of viable spores detected in the test based on the standard curves would also have to be related to the predicted level of disease in a particular soil type for the spore load estimation to be of practical use. Using a bait plant test in conjunction with the development of the real-time PCR test may be useful in comparing the predicted spore load measured to the disease level present. If a reliable test could be developed which could predict the disease severity that would be likely to occur, it would allow growers to determine quickly if their field was contaminated and to what extent, so that appropriate control measures against clubroot could be taken.

In the absence of a test that allows sophisticated estimates of the spore load in the soil, control treatments that are effective at controlling clubroot in a wide range of situations

are required. Glasshouse and field trials were therefore carried out in this study to examine the effectiveness of many conventional and novel treatments for providing sustainable, consistent and effective clubroot control. Treatments such as fungicides, limes, soil additives, biocontrol agents, surfactants and novel compounds such as waste products from the shellfish and mushroom growing industries were tested.

The only treatment to significantly reduce disease in both of the field trials carried out was Perlka (a granular form of the fertiliser calcium cyanamide). Other treatments that significantly reduced disease in the field were powdered calcium oxide, shell sand, powdered calcium carbonate, LimeX70 (sugarbeet waste lime), the fungicide Ranman (active ingredient cyazofamid) and the combination of the fungicide Shirlan (active ingredient fluazinam) and shell sand. With the exception of the two fungicides (Ranman and Shirlan) which specifically targeted oomycete fungi, and the fertiliser Perlka (which had fungistatic properties), the most effective treatments in the soil raised soil pH and calcium levels. These treatments were also highly effective at significantly reducing clubroot in the glasshouse trials with treatments such as LimeX70 and powdered calcium carbonate inhibiting disease completely.

Extractable calcium and soil pH measurements observed throughout the duration of the field trials suggested that fine powdered limes could react rapidly in the soil to increase pH and calcium to levels that were considered to be unsuitable for clubroot development. The results also suggested that adding treatments to the soil one day to two weeks before transplanting was sufficient to achieve high pH and calcium levels at planting which may play a role in disease reduction. Therefore, fine powdered lime applied close to transplanting may be the optimal strategy for effective clubroot control. Additional experiments examining the effect of different timings of addition of different types of lime to the soil may reveal the optimal combination of lime and addition date before transplanting to achieve maximum control. The field and glasshouse trials did not demonstrate that there was a specific pH or calcium level in the soil that would guarantee a significant reduction in clubroot, although treatments that raised pH above

7.3 and extractable calcium above 5900 mg/l in the glasshouse generally offered the greatest reductions in disease.

The literature on clubroot control suggested that pH and calcium act synergistically at controlling clubroot. Membrane permeability can be altered by high calcium and alkaline pH and this could affect intracellular phases of the pathogen during proliferation. An increase in pH and calcium could also strengthen plant cell walls thereby reducing invasion by the zoospores of the pathogen. A role for calcium channels in the hypersensitive response of resistant plants towards *P. brassicae* has also been hypothesised and therefore the addition of treatments that add calcium to the soil could have been mimicking the defence response of the plant host to provide control. The exact mechanism of the involvement of pH and calcium is still unclear and future research could investigate this in an attempt to develop greater control measures against clubroot. Stages in the *P. brassicae* life cycle where calcium could act are summarised in table 6.1 below.

Table 6.1. The potential effects that calcium has at controlling clubroot

Calcium effect	Where in the life cycle the controlling effect could take place	Reference
High soil calcium is required for structural needs	Rigidifying of plant cell wall and plasma membrane would stop the physical entry of zoospores into the host	Hepler, 2005
High soil calcium is required to allow tissues to absorb and retain solutes	Hosts would have better nutrition which would help prevent the development of clubroot in the roots by helping to produce defence compounds	Hepler, 2005
	The roots would have reduced leakage of compounds that may attract P. brassicae zoospores hence reducing zoospore entry into root hairs	White, 1998
	Calcium could affect the permeability of P. brassicae which may affect its ability to complete its life cycle	Moreno and Docampo, 2003
Calcium is required as a signalling molecule	Calcium induces the germination of resting spores and could cause the spores to germinate and die in the absence of a host	Yano *et al.,* 1991
	Calcium signalling is involved in gene expression in both the parasite and host so	Pickard and Ding, 1993

	different levels of calcium could affect the host/parasite interaction	
	Calcium signalling is involved in the differentiation of host cells so altering calcium levels could prevent hypertrophy and spread of disease	Voorrips, 1995
	Increased calcium is required for phenylalanine ammonia-lyase (PAL) activity which activates defence responses in resistant plants and prevents disease development in the roots	Takahashi *et al.,* 2002
Increased levels of calcium increases proton pumping	In sheared walls, calcium influx activates auxin porters. High calcium levels then saturate binding proteins responsible for enhancing auxin porter activity which would prevent auxin from increasing cell division hence decreasing disease spread	Pickard and Ding, 1993
Expanding cells require high levels of calcium	Calcium influx is mediated by ion channels in response to high levels of calcium. An increase in extracellular calcium decreases intracellular calcium which causes less root growth and therefore less disease spread	Kelling and Schulte, 2004
Influx of high levels of calcium results in cell death	In clubroot resistant plants, a hypersensitive response (cell death) occurs which reduces the spread of disease in the roots. Calcium is involved in some way in the induction of this HR	Takahashi *et al.,* 2006

Inconsistencies were found in the level of control achieved with the use of the same treatments in the two different field trials. Possible explanations for this included differences between the two field sites in disease pressure, spore race (as determined by an ECD test), environmental conditions, initial soil pH and nutrient status, and the times of addition of the treatments. The disease pressure and the environmental conditions may have been the most critical factors in the differences between the results of the two trials. The second field trial may have had a higher disease pressure and the weather conditions were much warmer and wetter. It was shown that fewer treatments reduced clubroot in this trial compared with the first trial where the weather conditions were milder, the initial spore load was possibly less and the initial soil pH was high. This would suggest that the effectiveness of treatments to control clubroot may vary with varying field conditions.

In the field trials, calabrese was grown on raised beds to increase soil drainage and promote good head growth despite the presence of clubroot in the soil. This appeared to be an effective strategy at maximising head weights of diseased plants under moderate rainfall conditions. Glasshouse experiments also demonstrated that it was generally not effective to apply powdered lime and treatments containing calcium to the outside of module compost before transplanting to control clubroot. This suggested that the whole root zone had to be in contact with a treatment to achieve good control. Therefore, in the field, treatments were applied in bands along the soil and were incorporated to a depth of 10 cm. This method of treatment addition did not adversely affect any of the plants and could possibly be an effective technique to use commercially when applying clubroot control treatments. This application technique effectively tripled the rate of the treatments added to the soil without increasing costs and also concentrated the treatments around the root zone. The use of raised beds and the strategic application of treatments could be used as part of an integrated management approach to reduce clubroot severity.

Adding combinations of treatments in attempt to illucidate an additive effect at controlling clubroot was shown to be generally ineffective in both the field and glasshouse trials with the exception of combinations of calcium carbonate and Borax, and QuickCal and Perlka in the glasshouse and Shirlan and shell sand in the field. Treatment combinations were not generally observed to be a beneficial strategy for clubroot control in this study.

The field and glasshouse results demonstrate that it is essential to know the initial spore load of the soil as this may influence the effectiveness of the treatments applied to the soil. Perlka was observed to be the most consistent treatment, providing significant disease reduction in the field and therefore may provide consistent control over a wide range of field conditions. The experiments also demonstrated that LimeX70 and powdered calcium oxide were the optimal lime products to use for control, possibly as a result of the rapid increase and highest levels of pH in the soil of any treatment tested.

The results also suggest that lime treatments may have to be applied between one day and two weeks in advance of transplanting to reach a pH and calcium level that was inhibitive of *P. brassicae*. They also demonstrated that adding the treatments in bands around the root ball in the soil, and growing the calabrese on raised beds may have reduced disease severity and enhanced the ability of the plants to overcome the detrimental effects caused by clubroot. All of these observations could provide useful information as to the best strategies for clubroot control.

Some treatments that were very effective at controlling disease in the glasshouse but were less effective in the field were SMC and Shirlan. The inconsistencies between glasshouse and field trial results may have been due to the environmental factors and natural soil biota present in the field that could not be controlled, unlike in the glasshouse. The glasshouse experiments did suggest that soil microflora had a part to play in the severity of clubroot. Treatments such as the broad spectrum fungicide Amistar increased disease in the glasshouse and had no effect on disease in the field. Therefore, knowledge of which microorganisms in the soil interact with *P. brassicae*, enhancing or inhibiting disease development, may be a necessary prerequisite to further research aiming to develop biocontrol agents for use against clubroot. The biocontrol agents tested in the glasshouse and field in this study were not effective at controlling disease and the lack of exact knowledge concerning how *P. brassicae* interacts with the soil microflora may have accounted for this.

Although the aim of this study was to develop clubroot control measures, some of the novel treatments tested such as Biohumate, oilseed rape meal and the broad-spectrum fungicide Amistar significantly increased disease. Oilseed rape meal increased clubroot severity in both the glasshouse and the field and may have been having an effect on resting spore germination. Treatments that enhanced the germination of resting spores could be useful as a control measure because spores could be encouraged to germinate in the absence of a host plant which may cause them to die naturally. Encouraging

germination and then adding a control treatment to destroy zoospores may also be an effective strategy to control clubroot.

The use of a calcium channel blocker such as peppermint oil, in addition to high rates of calcium carbonate, completely controlled disease in the glasshouse. This observation gave insights into the development of the disease, suggesting that calcium channels in either the host or pathogen are involved in clubroot development. Improved knowledge of the life-cycle of *P. brassicae* and factors that influence individual stages could help to develop treatments that are specifically targeted and which may therefore provide more consistently increased control than current control treatments.

Future methods to control clubroot could involve a fully integrated set of management techniques. A diagnostic test that not only quantified the level of spores in the soil (related to a predicted disease severity in the field), but which also detected specific races of the pathogen in the soil would be a critical first step in determining control measures. This first stage in the control strategy would allow specific control measures unique to a particular field to be carried out for maximum disease reduction and would also provide a cost-effective way to increase yields. The practice of growing brassicas on raised beds and targeting the application of treatments around the root zone could also be routinely practiced as an agronomic measure to reduce the level of disease development. Applying fine powdered limes such as LimeX70 or calcium oxide close to transplanting to increase soil pH and calcium may provide a good general method for reducing clubroot levels. Split applications of Perlka or treatments applied at optimal times before transplanting to achieve optimal control could also be determined and applied to the soil in a commercial environment. Some spore races may however become tolerant to treatments used on a regular basis due to natural selection. Therefore, treatment rotations or treatment combinations acting on different parts of the life-cycle could be envisaged as a way of providing sustainable control. The use of resistant cultivars of plants would also be a good strategy to achieve high levels of clubroot control. Cultivars that were not affected by the control treatments added to the

soil could also be used (Perlka is phytotoxic to certain cultivars, possibly due to the fact that causes boron deficiency (Williamson and Dyce, 1989). Host plants may also enhance the presence of certain pathotypes of *P. brassicae* in the soil due to different pathotypes preferentially infecting plants with particular genotypes (Crute *et al.,* 1983) and therefore different plant cultivars may have to be rotated in an attempt to prevent the build-up of virulent strains.

Further research required before such an integrated approach to clubroot control may be achieved would be to develop the diagnostic test to determine not only quantities of spores but also specific races. A wider range of novel treatments that could be added to the soil to give more control than is currently achieved could be developed. Treatments that were discovered to have a controlling effect in the glasshouse such as SMC could be optimised further in the glasshouse where variable factors could be controlled, then could be tested and optimised further in the field to provide evidence to growers that alternative, new and sustainable treatments could potentially be used in a control strategy against clubroot. Testing the effectiveness of a wide range of treatments on field soil in the glasshouse (which would remove the effect of the variable conditions of the field on treatment effectiveness) could increase the understanding of how treatments may act on *P. brassicae* development, and this knowledge could be used to optimise the treatments for field use. Standardised field trials may also be developed as a useful way of testing the effectiveness of control measures. Adding the same treatments at the same time before transplanting in different soil types with the same spore loads could give an indication of how consistent these treatments would be at providing control commercially and could determine standard methods for applying treatments to control clubroot in the field.

Conclusions

This study has highlighted possible treatments and farming practices that could be used to enhance clubroot control in the field. The development of the diagnostic test that was carried out in the study still requires considerable further work, but when fully developed would be a powerful tool for brassica growers in determining optimal control treatments for particular fields. Such a diagnostic test would also be a valuable research tool, allowing the rapid identification of effective treatments that destroyed resting spores. Knowledge of strains of clubroot present within the soil coming from a diagnostic test may influence the choice of control method. The control of clubroot is complex and more research is still needed to discover the mechanisms behind why treatments tested in this study controlled disease and how the soil and environmental factors in the field interacted with these treatments to alter their effectiveness across different sites.

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Appendix 1

Table 1. Effect of combinations of biohumate, calcium carbonate and potassium tetraborate on clubroot severity: cv. Marathon and Monaco (2004)

Treatment	Disease Index cv. Marathon	Disease Index Sig. probability	Disease Index cv. Monaco	Disease Index Sig. probability
Negative control	0	<0.001*	0	<0.001*
Positive control	55.6		64.8	
Biohumate	100	0.077*	19.4	<0.001*
Biohumate + $CaCO_3$	25	0.028*	22.2	<0.001*
Biohumate + Potassium tetraborate	41.7	0.183	69.4	0.345
Biohumate + $CaCO_3$ + Potassium tetraborate	50	0.373	33.3	<0.001*
Potassium tetraborate	75	0.656	61.1	0.104
Potassium tetraborate + $CaCO_3$	25	0.028*	50	0.005*

*P<0.05

Table 2: Effect of combinations of biohumate, calcium carbonate and potassium tetraborate on plant fresh weight: cv. Marathon and Monaco (2004)

Treatment	Fresh weight (g), cv. Marathon	Fresh weight SEM	Fresh weight Sig. probability	Fresh weight (g), cv. Monaco	Fresh weight SEM	Fresh weight Sig. probability
Negative control	19.9	± 5.36	0.919	24.7	± 3.03	0.959
Positive control	23.6	± 3.11		18.1	1 ± 3.02	
Biohumate	17.8	± 8.95	0.796	12.6	± 2.83	0.062
Biohumate + CaCO3	23.3	± 10.5	0.843	10.4	± 2.4	0.026*
Biohumate + Potassium tetraborate	31.2	± 12.6	0.392	21.5	5 ± 4.26	0.533
Biohumate + CaCO3 + Potassium tetraborate	30.2	± 17.4	0.438	16.8	± 4.03	0.223
Potassium tetraborate	28.7	± 8.29	0.517	15.5	0.157	0.157
Potassium tetraborate + CaCO3	34.3	± 10.17	0.267	22.6	± 3.41	0.639

LSD FW = 11.25, *P<0.05

Table 3. Effect of surfactants on clubroot severity: cv. Marathon and Monaco (2004)

Treatment	Disease Index cv. Marathon	Disease Index Sig. probability	Disease Index cv. Monaco	Disease Index Sig. probability
Negative control	0	<0.001*	0	<0.001*
Positive control	55.6		64.8	
Agral	66.7	0.323	52.8	0.019*
Quillaja	75	0.656	33.3	0.002*
Yucca	25	0.028*	30.6	0.002*

*P<0.05

Table 4. Effect of surfactants on plant fresh weight: cv. Marathon and Monaco (2004)

Treatment	Fresh weight (g), cv. Marathon	Fresh weight SEM	Fresh weight Sig. probability	Fresh weight (g), cv. Monaco	Fresh weight SEM	Fresh weight Sig. probability
Negative control	19.9	± 5.36	0.919	24.7	± 3.03	0.959
Positive control	23.6	6 ± 3.11		18.1	± 3.02	
Agral	20.9	± 7.54	0.984	19.8	± 4.86	0.429
Quillaja	29.7	± 8.25	0.463	19.4	± 2.31	0.428
Yucca	13.8	± 1.6	0.553	16.1	± 3.38	0.269

LSD FW = 11.25, *P<0.05

Table 5. Effect of biocontrol agents on clubroot: cv. Marathon and Monaco (2004)

Treatment	Disease Index cv. Marathon	Disease Index Sig. probability	Disease Index cv. Monaco	Disease Index Sig. probability
Negative control	0	<0.001*	0	<0.001*
Positive control	55.6		64.8	
Bactolife DP104	25	0.028*	11.1	<0.001*
Bactolife S	16.7	0.009*	11.1	<0.001*
Chitin	25	0.028*	38.9	0.007*
P. chlororaphis	41.7	0.183	30.6	0.021*
P. fluorescens	33.3	0.077	44.4	0.183

*P<0.05

Table 6. Effect of biocontrol agents on plant fresh weight: cv. Marathon and Monaco (2004)

Treatment	Fresh weight (g), cv. Marathon	Fresh weight SEM	Fresh weight Sig. probability	Fresh weight (g), cv. Monaco	Fresh weight SEM	Fresh weight Sig. probability
Negative control	19.9	± 5.36	0.919	24.7	± 3.03	0.959
Positive control	23.6	± 3.11		18.1	± 3.02	
Bactolife DP104	14.3	± 7.97	0.580	2.7	± 0.86	0.001*
Bactolife S	17.9	± 7.61	0.804	2.9	± 1.11	0.001*
Chitin	20.6	± 4.38	0.982	21.2	± 3.07	0.522
P. chlororaphis	17.9	± 2.81	0.532	13.7	± 2.34	0.584
P. fluorescens	30.3	± 5.53	0.660	15.2	± 1.86	0.668

LSD FW = 11.25, *P<0.05

Table 7. Effect of treatments containing calcium on clubroot severity: cv. Marathon and Monaco (2004)

Treatment	Disease Index cv. Marathon	Disease Index Sig. probability	Disease Index cv. Monaco	Disease Index Sig. probability
Negative control	0	<0.001*	0	<0.001*
Positive control	55.6		64.8	
Ca carbonate	41.7	0.183	8.3	<0.001*
Ca oxide	83.3	0.373	80.6	0.776
Ca silicate	58.3	0.267	52.8	0.009*
Perlka	41.7	0.183	8.3	<0.001*

*P<0.05

Table 8. Effect of treatments containing calcium on plant fresh weight: cv. Marathon and Monaco (2004)

Treatment	Fresh weight (g), cv. Marathon	Fresh weight SEM	Fresh weight Sig. probability	Fresh weight (g), cv. Monaco	Fresh weight SEM	Fresh weight Sig. probability
Negative control	19.9	± 5.36	0.919	24.7	± 3.03	0.959
Positive control	23.6	± 3.11		18.1	± 3.02	
Ca carbonate	34.8	± 8.53	0.249	26.5	± 4.86	0.864
Ca oxide	28.4	± 7.38	0.532	26.8	± 3.07	0.877
Ca silicate	54.4	± 17.27	0.006*	23.0	± 4.95	0.666
Perlka	30.8	± 11.87	0.411	16	± 3.05	0.182

LSD FW = 11.25, *P<0.05

Table 9. Effect of fungicides on clubroot severity: cv. Marathon and Monaco (2004)

Treatment	Disease Index cv. Marathon	Disease Index Sig. probability	Disease Index cv. Monaco	Disease Index Sig. probability
Negative control	0	<0.001*	0	<0.001*
Positive control	55.6		64.8	
Ranman	0	<0.001*	2.8	<0.001*
Shirlan	0	<0.001*	13.9	<0.001*

*P<0.05

Table10. Effect of fungicides at on clubroot severity: cv. Marathon and Monaco (2004)

Treatment	Fresh weight (g), cv. Marathon	Fresh weight SEM	Fresh weight Sig. probability	Fresh weight (g), cv. Monaco	Fresh weight SEM	Fresh weight Sig. probability
Negative control	19.9	± 5.36	0.919	24.7	± 3.03	0.959
Positive control	23.6	± 3.11		18.1	± 3.02	
Ranman	10.2	± 2.68	0.375	24.3	± 4.24	0.674
Shirlan	30.1	± 12.71	0.443	17	± 4.63	0.465

LSD FW = 11.25, *P<0.05

Table 11. Effect of biocontrol agents on clubroot severity and plant fresh weight: cv. Monaco (2005)

Treatment	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	0		<0.001*	80	± 9.24	0.179
Positive control	60.4	± 7.95		61.9	± 9.43	
Bactolife DP104	35.5	± 11.58	0.124	68.9	± 9.59	0.553
Bactolife S	56.3	± 14.17	0.124	70.9	± 11.39	0.464
P. chlororaphis 104 CFU/ml	44.6	± 13.85	0.086	69.7	± 6.67	0.507
P. chlororaphis 106 CFU/ml	46.2	± 12.51	0.106	73.2	± 10.42	0.336
P. chlororaphis 108 CFU/ml	55.6	± 11.82	0.213	57.1	± 12.94	0.456
P. fluorescens 104 CFU/ml	45.1	± 12.45	0.066	73.1	± 10.90	0.346
P. fluorescens 106 CFU/ml	50.3	± 8.95	0.474	57.9	± 6.92	0.735
P. fluorescens 108 CFU/ml	77.3	± 8.92	0.862	66.7	± 10.07	0.682
Shell sand	0.1	± 0.1	<0.001*	63.1	± 9.23	0.914
SMC 10%	55.4	± 10.4	0.862	78	± 15.05	0.170

SMC 30%	3.8	± 2.72	<0.001*	72.1	± 11.03	0.384

FW: LSD = 27.2, *P<0.05

Table 12. Effect of plant extracts and liquid chitosan on clubroot severity and plant fresh weight: cv. Monaco (2005)

Treatment	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	0		<0.001*	80	± 9.24	0.179
Positive control	60.4	± 7.95		61.9	± 9.43	
Biomagic	27.2	± 11.22	0.075	67.4	± 11.34	0.641
Peppermint oil	43.4	± 12.92	0.479	50	± 5.70	0.352
Rhubarb leaves	39.4	± 10.96	0.130	73.2	± 12.33	0.334
Rhubarb water	43.4	± 12.53	0.196	60	± 9.71	0.875
Softguard	51.9	± 10.71	0.418	55	± 11.16	0.585

FW: LSD = 27.2, *P<0.05

Table 13. Effect of combinations of Borax, calcium carbonate and Perlka on clubroot severity and plant fresh weight: cv. Monaco (2005)

Treatment	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	0		<0.001*	80	± 9.24	0.179
Positive control	60.4	± 7.95		61.9	± 9.43	
Borax	24.7	± 12.25	0.033*	58.1	± 6.54	0.749
Borax +CaCO3	0.7	± 0.08	0.002*	70.5	± 8.66	0.462
Borax +CaCO3 + Perlka	0	0	<0.001*	5.4	± 1.614	<0.001*

FW: LSD = 27.2, *P<0.05

Table 14. Effect of Perlka and different types of limes on clubroot severity and plant fresh weight: cv. Monaco (2005)

Treatment	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	0		<0.001*	80	± 9.24	0.179
Positive control	60.4	± 7.95		61.9	± 9.43	
Ca carbonate	8.1	± 6.2	0.004*	74.2	± 9.63	0.294
CaCO3 +Perlka	0	0	<0.001*	13.8	± 5.68	<0.001*
Ca oxide	0	0	<0.001*	68.5	± 10.24	0.571
CaO + Perlka	0	0	<0.001*	32.4	± 8.86	0.013*
LimeX70	0	0	<0.001*	79.7	± 9.10	0.741
Perlka	0	0	<0.001*	4	± 1.42	<0.001*

FW: LSD = 27.2, *P<0.05

Table 15. Effect of surfactants on clubroot severity and plant fresh weight: cv. Monaco (2005)

Treatment	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	0		<0.001*	80	± 9.24	0.179
Positive control	60.4	± 7.95		61.9	± 9.43	
Quillaja	44	± 9.62	0.393	55.4	± 10.83	0.583
Yucca	28.2	± 10.66	0.126	70.8	± 11.85	0.449

FW: LSD = 27.2, *P<0.05

Table 16. Effect of fungicides on clubroot severity and plant fresh weight: cv. Monaco (2005)

Treatment	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	0		<0.001*	80	± 9.24	0.6179
Positive control	60.4	± 7.95		61.9	± 9.43	
Ranman	6.4	± 6.06	<0.007*	80.9	± 11.06	0.106
Shirlan	0.0008	± 0.001	<0.001*	76.1	± 11.26	0.227

FW: LSD = 27.2, *P<0.05

Table 17. Effect of Amistar and *P. chlororaphis* on clubroot severity, plant fresh weight and final soil pH: cv. Monaco (2006)

Treatment	Final soil pH	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	6.6	0		<0.001*	16.32	± 2.62	0.846
Positive control	6.6	6.3	± 7.34		15.79	± 2.43	
Amistar	6.3	32.1	± 10.51	0.496	13.14	±2.77	0.408
Amistar + P. chlororaphis	6.4	42.3	± 10.82	0.976	11.72	± 2.76	0.197

pH: LSD = 0.4; FW: LSD = 6.94; % disease LSD = *P<0.05

Table 18. Effect of biocontrol agents on clubroot severity, plant fresh weight and final soil pH: cv. Monaco (2006)

Treatment	Final soil pH	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	6.6	0		<0.001*	16.32	± 2.62	0.846
Positive control	6.6	6.3	± 7.34		15.79	± 2.43	
Bactolife DP104	6.4	3.9	± 0.64	0.424	16.32	± 4.01	0.920
P. chlororaphis	6.4	3	± 1.23	0.080	14.61	± 2.56	0.685
SMC 30 %	7.1*	5.5	± 1.04	0.127	19.46	± 3.89	0.432
SMC + P. chlororaphis	7.0	2.7	± 1.18	0.469	20.57	± 2.32	0.162

pH: LSD = 0.4; FW: LSD = 6.94; % disease LSD = 2.02; *P<0.05

Table 19. Effect of selected treatments that add nutrients to the soil on clubroot severity, plant fresh weight and final soil pH: cv. Monaco (2006)

Treatment	Final soil pH	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	6.6	0		<0.001*	16.32	± 2.62	0.846
Positive control	6.6	6.3	± 7.34		15.79	± 2.43	
Bod Ayre seaweed	6.3	4.2	± 0.86	0.424	20.87	± 2.77	0.934
Ca nitrate	6.2	14.5	± 5.17	0.175	26.61	± 3.11	0.004*
Copper carbonate	6.8	6.7	± 4.38	0.209	14.18	± 3.82	0.441
Gypsum	6.3	10.9	± 4.34	0.794	13.87	± 2.17	0.885

pH: LSD = 0.4; FW: LSD = 6.94; % disease LSD = 1.64; *P<0.05

Table 20. Effect of combinations of Borax, calcium carbonate and P. chlororaphis on clubroot severity, plant fresh weight and final soil pH: cv. Monaco (2006)

Treatment	Final soil pH	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	6.6	0		<0.001*	16.32	± 2.62	0.846
Positive control	6.6	6.3	± 7.34		15.79	± 2.43	
Borax	7.0	5.2	± 1.41	0.776	15.77	± 2.45	0.978
Borax + CaCO3	7.4*	0.5	± 0.49	0.034*	17.41	± 2.76	0.685
Borax + P. chlororaphis	6.8	21.4	± 8.15	0.853	14.6	± 3.49	0.620
Borax + CaCO3 + P. chlororaphis	7.3*	0		<0.001*	13.72	± 3.49	0.524

pH: LSD = 0.4; FW: LSD = 6.94; % disease LSD = 2.66; *P<0.05

Table 21. Effect of combinations of calcium carbonate, Amistar and *P. chlororaphis* on clubroot severity, plant fresh weight and final soil pH: cv. Monaco (2006)

Treatment	Final soil pH	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	6.6	0		<0.001*	16.32	± 2.62	0.846
Positive control	6.6	6.3	± 7.34		15.79	± 2.43	
Ca carbonate	7.1*	0.1	± 0.1	0.033*	14.3	± 1.75	0.763
CaCO3 + Amistar	7.3*	1.7	± 1.09	0.127	14.29	± 2.98	0.650
CaCO3 + P. chlororaphis	7.3*	4.8	± 4.79	0.080	14.5	± 2.11	0.737
CaCO3 + Amistar + P. chlororaphis	7.3*	1.6	± 0.8	0.037*	15.52	± 2.95	0.848
CaCO3 + CaO	7.4*	0		<0.001*	17.06	± 1.66	0.622

pH: LSD = 0.4; FW: LSD = 6.94; % disease LSD = 2.66; *P<0.05

Table 22. Effect of combinations of calcium oxide, Shirlan and *P. chlororaphis* on clubroot severity, plant fresh weight and final soil pH: cv. Monaco (2006)

Treatment	Final soil pH	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	6.6	0		<0.001*	16.32	± 2.62	0.846
Positive control	6.6	6.3	± 7.34		15.79	± 2.43	
Ca oxide	7.7*	0	0	<0.001*	17.00	± 2.69	0.460
CaO + Shirlan	7.5*	0	0	<0.001*	16.18	± 2.13	0.683
CaO + P. chlororaphis	7.4*	1	± 0.58	0.079	15.97	± 2.34	0.921
CaO + Shirlan + P. chlororaphis	7.3*	0	0	<0.001*	16.84	± 3.65	0.863

pH: LSD = 0.4; FW: LSD = 6.94, *P<0.05

Table 23. Effect of combinations of LimeX, Shirlan, and *P. chlororaphis* on clubroot severity, plant fresh weight and final soil pH: cv. Monaco (2006)

Treatment	Final soil pH	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	6.6	0		<0.001*	16.32	± 2.62	0.846
Positive control	6.6	6.3	± 7.34		15.79	± 2.43	
LimeX	7.4*	0		<0.001*	18.05	± 2.24	0.478
LimeX + Shirlan	7.5*	0		<0.001*	18.06	± 3.88	0.682
LimeX + P. chlororaphis	7.4*	0		<0.001*	16.25	± 3.58	0.993
LimeX + Shirlan + P. chlororaphis	7.6*	0		<0.001*	20.27	± 4.64	0.356

pH: LSD = 0.4; FW: LSD = 6.94, *P<0.05

Table 24. Effect of Perlka and QuickCal on clubroot severity, plant fresh weight and final soil pH: cv. Monaco (2006)

Treatment	Final soil pH	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	6.6	0		<0.001*	16.32	± 2.62	0.846
Positive control	6.6	6.3	± 7.34		15.79	± 2.43	
Perlka	6.9	5.7	± 2.77	0.331 LSD = 1.64	26.01	± 2.93	0.006*
Perlka + QuickCal	8.9*	0		<0.001*	25.13	± 5.03	0.020*
QuickCal	7.4*	3.7	± 2.36	0.399 LSD = 1.57	14.75	± 2.09	0.829

pH: LSD = 0.4; FW: LSD = 6.94, *P<0.05

Table 25. Effect of Peppermint, SIPeco and Softguard on clubroot severity, plant fresh weight and final soil pH: cv. Monaco (2006)

Treatment	Final soil pH	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	6.6	0		<0.001*	16.32	± 2.62	0.846
Positive control	6.6	6.3	± 7.34		15.79	± 2.43	
Peppermint	6.8	27.7	± 8.17	0.987 LSD = 1.51	12.94	± 2.73	0.378
Peppermint + CaCO3	7.5*	0		<0.001*	15.55	± 2.05	0.979
SIPeco	6.7	32.8	± 9.79	0.992 LSD = 1.51	12.37	± 2.12	0.337
Softguard	6.6	6.0	± 4.38	0.461 LSD = 1.55	12.17	± 2.80	0.385

pH: LSD = 0.4; FW: LSD = 6.94, *P<0.05

Table 26. Effect of combinations of Quillaja, calcium carbonate and *P. chlororaphis* on clubroot severity, plant fresh weight and final soil pH: cv. Monaco (2006)

Treatment	Final soil pH	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	6.6	0		<0.001*	16.32	± 2.62	0.846
Positive control	6.6	6.3	± 7.34		15.79	± 2.43	
Quillaja	6.6	17.9	± 8.52	0.164 LSD = 1.40	13.67	± 0.91	0.676
Quillaja + CaCO3	7.6*	3.9	± 4.73	0.182 LSD = 1.51	14.37	± 2.75	0.665
Quillaja + P. chlororaphis	6.5	12.7	± 9.83	0.869 LSD = 1.51	11.41	± 1.80	0.225
Quillaja + CaCO3 + P. chlororaphis	7.1*	19.3	± 6.16	0.326 LSD = 1.57	12.25	± 3.24	0.224

pH: LSD = 0.4; FW: LSD = 6.94, *P<0.05

Table 27. Effect of combinations of shell sand, Shirlan and *P. chlororaphis* on clubroot severity, plant fresh weight and final soil pH: cv. Monaco (2006)

Treatment	Final soil pH	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	6.6	0		<0.001*	16.32	± 2.62	0.846
Positive control	6.6	6.3	± 7.34		15.79	± 2.43	
Shell sand	7.3*	0.3	± 0.43	0.071	14.41	± 2.64	0.642
Shell sand + P. chlororaphis	7.3*	0		<0.001* LSD = 2.08	15.59	± 3.21	0.795
Shell sand + Shirlan	7.0	0.18	± 0.15	0.073	18.48	± 3.05	0.438
Shell sand + Shirlan + P. chlororaphis	7.4*	0.1	± 0.08	0.073 LSD = 2.06	14.14	± 2.41	0.656

pH: LSD = 0.4; FW: LSD = 6.94, *P<0.05

Table 28. Effect of combinations of Shirlan, calcium carbonate and *P. chlororaphis* on clubroot severity, plant fresh weight and final soil pH: cv. Monaco (2006)

Treatment	Final soil pH	% disease	% disease SEM	% disease Sig. probability	Fresh weight (g)	Fresh weight SEM	FW Sig. probability
Negative control	6.6	0		<0.001*	16.32	± 2.62	0.846
Positive control	6.6	6.3	± 7.34		15.79	± 2.43	
Shirlan	6.8	0.4	± 0	<0.001*	15.28	± 3.83	0.733
Shirlan + CaCO3	7.3*	0		<0.001*	16.83	± 2.78	0.777
Shirlan + P. chlororaphis	6.9	0.2	± 0.23	0.033* LSD = 2.66	14.35	± 1.75	0.766
Shirlan + CaCO3 + P. chlororaphis	7.4*	0.5	± 0.22	0.033* LSD = 2.66	15.14	± 2.55	0.796

pH: LSD = 0.4; FW: LSD = 6.94; *P<0.05

Table 29. The addition of oilseed rape meal to clubroot inoculated pots of soil 8, 4, 2 and 0 weeks in advance of transplanting of calabrese cv. Monaco: its effect on disease level, fresh weight and root weight of the plants

No. of weeks OSR meal added in advance	% Disease; SEM	% Disease t pr.	Fresh weight (g); SEM	Fresh weight t pr.	Root weight (g); SEM	Root weight t pr.
Negative control	0	<0.001*	24.0 ± 1.94	0.581	4.64 ± 0.42	0.382
Positive control	26 ± 6.71		22.96 ± 1.85		5.48 ± 0.67	
0 weeks	16.7 ± 2.93	0.060	31.81 ± 2.88	<0.001*	6.00 ± 1.07	0.657
2 weeks	34.6 ± 10.89	0.216	30.82 ± 1.4	0.002*	6.88 ± 1.29	0.236
4 weeks	44.7 ± 6.34	0.011*	36.67 ± 1.21	<0.001*	9.07 ± 0.73	0.005*
8 weeks	44.9 ± 3.43	0.010*	33.24 ± 1.99	0.001*	8.24 ± 1.28	0.024*

LSD % disease = 14.05
LSD fresh weight = 4.70
LSD root weight = 2.38
*P<0.05

Table 30. The effect of adding calcium carbonate, calcium oxide and shell sand to the outside of module compost on disease and fresh weight of calabrese cv. Monaco modules planted into clubroot inoculated pots of soil

Treatment	% Disease; SEM	% Disease t pr.	Fresh weight (g); SEM	Fresh weight t. pr	Root weight (g); SEM RW	Root weight t pr.
Negative control	0	0.002*	29.28 ± 3.96	0.381	6.4 ± 0.96	0.491
Positive control	32.9 ± 7.77		25.22 ± 3.05		7.48 ± 0.99	
Ca carbonate	33.0 ± 8.99	0.987	23.24 ± 1.71	0.905	7.81 ± 1.08	0.830
Ca oxide	8.6 ± 5.55	0.018*	24.46 ± 7.11	0.809	5.43 ± 1.47	0.195
Shell sand	45.3 ± 8.46	0.215	24.52 ± 3.18	0.929	5.49 ± 0.86	0.209

LSD % Disease = 19.89
LSD Fresh weight = 12.22
LSD Root weight = 3.13
*P<0.05

Appendix 2

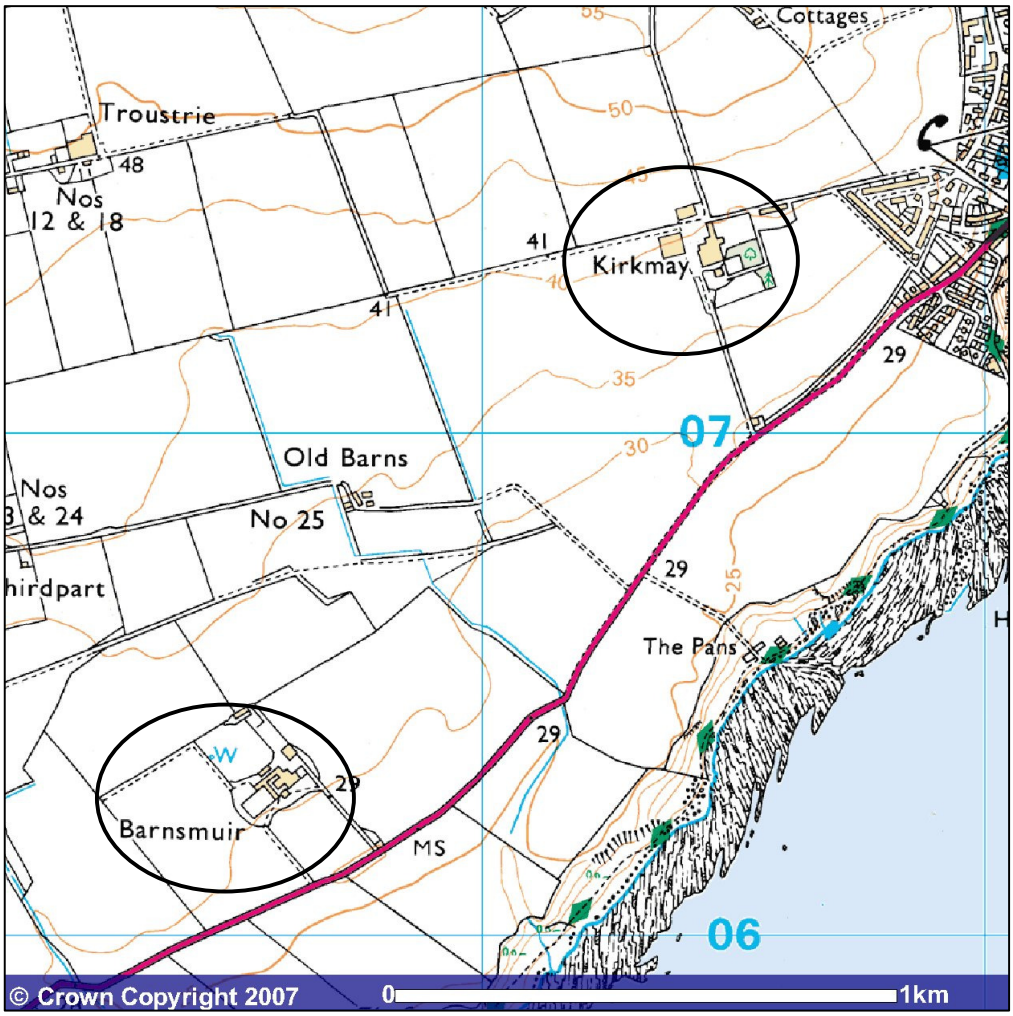

Image produced from the Ordnance Survey Get-a-map service. Image reproduced with kind permission of Ordnance Survey and Ordnance Survey of Northern Ireland.

Figure 1. Location of the Fife field trials

0	5 metres	5 metres	5 metres	5 metres	5 metres	5 metres
1	11	6	11	4	8	4
2	14	12	9	12	1	14
3	5	2	13	3	10	6
4	1	7	1	14	3	2
5	9	8	10	8	5	13
6	3	10	6	2	11	7
7	13	4	5	7	9	12
8	3	10	11	12	10	6
9	8	9	5	2	14	5
10	1	6	1	10	4	12
11	7	4	6	3	7	2
12	5	2	9	4	11	8
13	13	14	7	8	1	13
14	11	12	14	13	3	9

Double lines indicate treatment blocks. Plots are 5m x 1.8 m. Numbers within plots correspond to the position of the treatment with the same number (table 4.6).

Figure 2. Field trial layout, Kirkmay, Crail, Fife, 2005

	5 metres	5 metres	5 metres	5 metres	5 metres	5 metres
1	12	15	16	2	14	7
2	4	7	1	5	1	16
3	1	13	7	8	9	5
4	9	2	11	12	15	10
5	10	14	3	13	3	6
6	11	3	4	6	4	2
7	6	8	14	9	11	8
8	5	16	15	10	13	12
9	11	9	12	9	15	8
10	16	5	8	6	1	10
11	1	12	3	11	4	16
12	13	2	14	13	5	3
13	4	10	4	2	7	2
14	8	14	15	5	13	12
15	7	15	1	7	14	6
16	6	3	10	16	9	11

Double lines indicate treatment blocks. Plots are 5m x 1.8 m. Numbers within plots correspond to the position of the treatment with the same number (table 4.7).

Figure 3. Field trial layout, Barnsmuir, Crail, Fife, 2006

Table 1. Analysis of calcium carbonate (TruCarb295)

Typical Particle Size Analysis			Typical Chemical Analysis		% Mass
Sieve Aperture	% Passing	Specification Limits	Calcium	As CaO	55.7
710 µm	100		Magnesium	As MgO	0.18
425 µm	99.99	99.9 minimum	Aluminium	As Al2O3	0.06
212 µm	99.75	99 minimum	Iron	As Fe2O3	0.03
150 µm	99		Silica	As SiO2	0.3
75 µm	94	92 minimum	Loss on Ignition@ 1000Ec	As H2O	43.5
45 µm	82		Moisture		0.05
3 µm	17	15 – 25	Calcium carbonate	As CaCO3	99
Specific surface area 4000 cm2/g			Neutralising value	As CaO	56.9

Calcium carbonate (TruCarb295) is an off-white general purpose carboniferous limestone powder. The product is crushed and screened several times and then ground and air classified to produce a material of high purity. It is dried to give low moisture content. Source: Ballidon Quarry, Nr. Ashbourne, Derbyshire.

Table 2. Analysis of calcium oxide (Calbux 90M)

Typical Particle Size Analysis			Typical Chemical Analysis	% Mass
Sieve Aperture	% Passing	Specification Limits	CaO	94
1.2 mm	100	100 minimum	CaCO3	3.9
75 µm	92	90 minimum	MgO	0.5
53 µm	80		CaSO4	0.05
			Combined Moisture	0.8
Bulk Density	840-1020 kg/m3		SiO2	0.8
			Al2O3	0.1
Reactivity			Fe2O3	0.06
2 min	67oC	56 oC	Mn	178 ppm
Final	69		F	73 ppm
			Pb	2.6 ppm
			As	0.2 ppm
			Neutralising value (as CaO)	96.3

Calcium oxide (Calbux 90M) is a high purity, high reactivity quicklime. Available as fine, dry, white ground powder it is used in a wide cultivar of industrial processes. The process for making it involves calcining very pure processed limestone at high

temperatures after which the lime is crushed, screened and ground to the required grading profile. Source: It is manufactured from high-purity carboniferous limestone at Tunstead, near Buxton, Derbyshire.

Table 3. Analysis of LimeX70

Typical Particle Size Analysis		Typical Chemical Analysis	% Mass
Sieve Aperture	% Passing	CaCO3	52
5 mm	99	Organic material	15
3.35 mm	97	Silicates	5
150 µm	85	Water	30
		P2O5	10 kg/t
		MgO	7 kg/t
		SO3	5 kg/t
		Neutralising value	22%

LimeX70 is a precipitated calcium carbonate product from the British sugar beet process. Available as an off-white to dark grey friable material it is made of four main components: $CaCO_3$, organic material containing plant nutrients, inert silicates and water.

Table 4. Analysis of SMC and Shell sand treatments used in the trials

Determination	Results		Units
	Shell sand	Spent mushroom compost	
Dry Matter	99.7	31.5	%
Nitrogen	0.08	0.03	% DM
Total phosphate (P2O5)	0.09	0.01	%
Total potash (K2O)	0.01	0.03	%
Aqua regia calcium	31.3	0.07	% DM
Aqua regia magnesium	0.07	0.07	% DM
Aqua regia sodium	0.41	0.003	% DM
Aqua regia zinc	<15.2	273	mg/kg DM
Aqua regia copper	<3.34	46	mg/kg DM
Aqua regia iron	<113	2153	mg/kg DM
Aqua regia manganese	12.3	376	mg/kg DM
Aqua regia aluminium	94.7		mg/kg DM
Aqua regia sulphur	1520	0.16	mg/kg DM

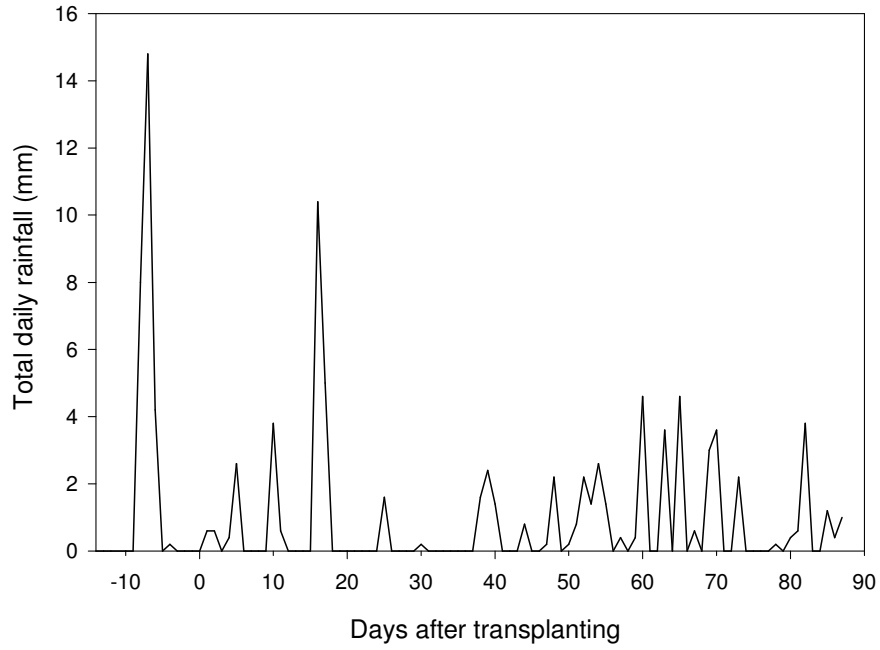

Figure 4. Daily rainfall (mm) over the course of the 2005 trial

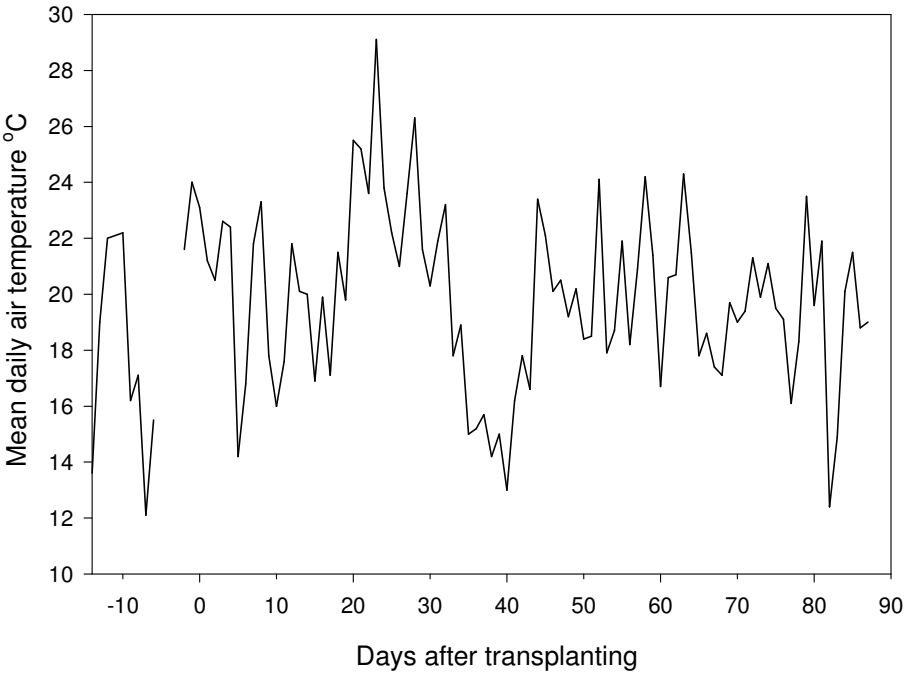

Figure 5. Mean daily temperature over the course of the 2005 trial

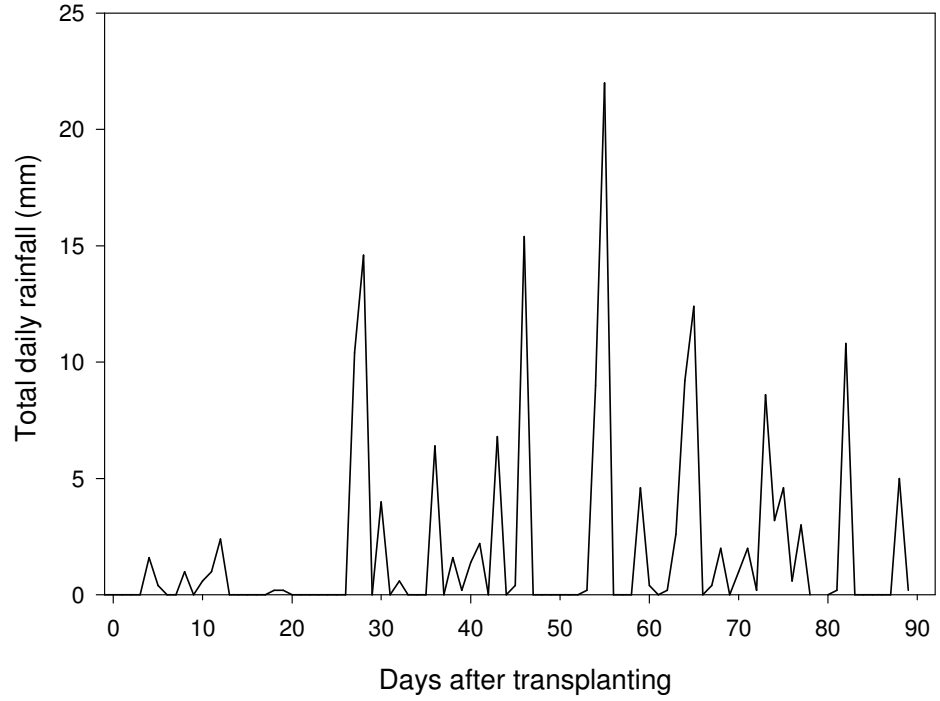

Figure 6. Daily rainfall (mm) over the course of the 2006 trial

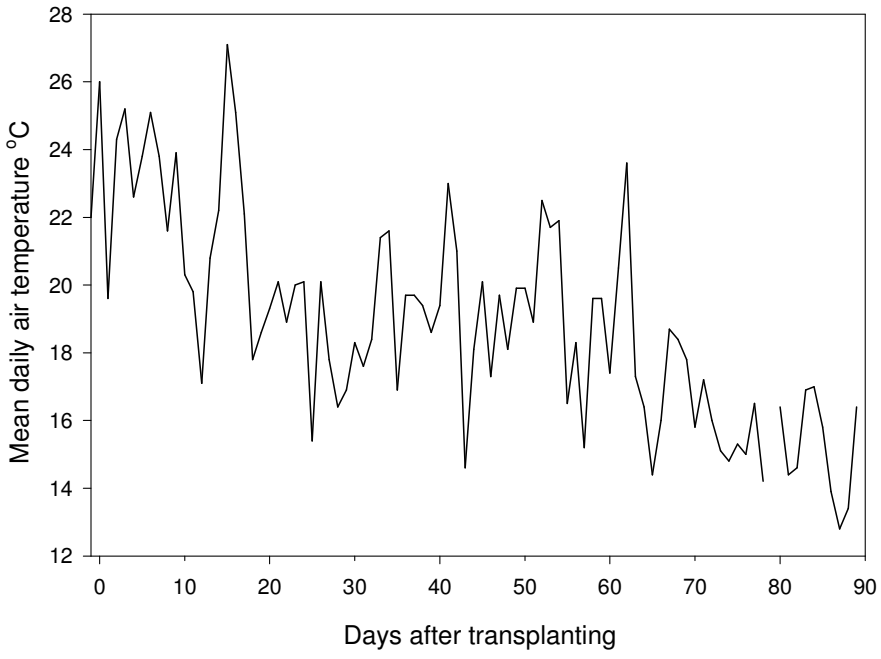

Figure 7. Mean daily temperature over the course of the 2006 trial

Table 5. SEM of 2006 pH measurements corresponding to table 5.11 in chapter 5

	SEMs of soil pH values sampled on selected days after transplanting					
Treatment	0	3	11	28	56	84
Control 1	± 0.06	±0.03	±0.03	±0.03	±0.09	±0.06
Control 2	± 0.05	± 0.04	± 0.06	± 0.05	± 0.09	± 0.09
Calcium carbonate	± 0.08	± 0.02	± 0.05	± 0.02	± 0.07	± 0.06
Calcium oxide	± 0.2	± 0.24	± 0.07	± 0.05	± 0.09	± 0.03
LimeX	± 0.09	± 0.05	± 0.07	± 0.02	± 0.09	± 0.03
Perlka	± 0.1	± 0.04	± 0.05	± 0.1	± 0.05	± 0.09
QuickCal	± 0.13	± 0.4	± 0.06	± 0.06	± 0.1	± 0.11
Shell sand	± 0.05	± 0.05	± 0.05	± 0.04	± 0.09	± 0.04
SMC	± 0.07	± 0.07	± 0.04	± 0.05	± 0.08	± 0.03

Table 6. SEM of 2006 extractable calcium measurements corresponding to table 5.12 in chapter 5

	SEMs of soil extractable calcium values sampled on selected days after transplanting					
Treatment	0	3	11	28	56	84
Control 1	± 28	± 24	± 31	± 16	± 47	± 18
Control 2	± 34	± 41	± 46	± 43	± 35	± 55
Calcium carbonate	± 557	± 674	± 759	± 460	± 551	± 898
Calcium oxide	± 537	± 893	± 554	± 1114	± 836	± 229
LimeX	± 325	± 603	± 476	± 542	± 708	± 275
Perlka	± 24	± 34	± 38	± 74	± 108	± 166
QuickCal	± 121	± 443	± 128	± 154	± 83	± 202
Shell sand	± 199	± 310	± 226	± 260	± 314	± 139
SMC	± 167	± 154	± 28	± 104	± 134	± 64

Table 7: Chitin composition

Protein %	Chitin %	Inorganic %	Lipids %
13.2	72.3	14.2	0.3

Metal	ppm
Ca	1741
Mg	8.92
Na	27.15
Sr	21.25
K	3.93
Fe	13.93
Mn	2.52
Sn	4.41
Si	3.6
Al	10.04
B	2.24
Zn	1.64
Se	0.62
Cu	0.74
Ba	0.74
Pb	0.51
Cr	0.73
Ti	0.1
Ni	0.36
Co	0.02
Cd	0.01